

AN INVESTIGATION INTO THE EFFECTS OF THE EPIDERMAL  
GROWTH FACTOR RECEPTOR TYROSINE KINASE INHIBITOR  
“GEFITINIB” ON HUMAN BREAST CANCER

By

Eleanor Gutteridge

BMedSci Nottingham 1993, BMBS Nottingham 1995,

FRCS (Ed) Gen Surg 2009

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# 1 Abstract

**Title** – An Investigation In to the Effects of the Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor “Gefitinib” on Human Breast Cancer

**Background** - In vitro studies have shown that ER+ acquired tamoxifen resistant MCF7 breast cancer cell lines can show elevated levels of EGFR expression with an increase in its subsequent signalling pathway(s) and that these are growth inhibited by gefitinib, an EGFR tyrosine kinase inhibitor. This thesis examines the effect of gefitinib on tamoxifen resistant human breast cancer in the clinical setting and in an ‘in-vivo’ mouse model.

**Patients and Methods** – This phase 2 clinical study recruited 54 patients. 28 were oestrogen receptor positive and had progressed on tamoxifen treatment (acquired resistance), the other 26 (48.1%) were oestrogen receptor negative (de novo resistance). Patients were given a loading dose of 1000mg gefitinib on Day 1 and then gefitinib 500mg as a once daily oral dosing until evidence of disease progression. Clinical data were recorded. Sequential tumour biopsies were taken pre-treatment, after 8 weeks therapy and at the development of resistance and analysed immunocytochemically to identify predictive factors for response to treatment and also to see the effect of treatment and resistance on tumour biology, encompassing monitoring steroid receptors, EGFR, HER2 and IGFR, downstream kinases MAPK and AKT, and the proliferation marker Ki67. In parallel with the clinical study, ER+ acquired tamoxifen resistant MCF7 xenografts (TAMR) were grown in nude mice in the presence of tamoxifen and treated with gefitinib 50mg per day orally (designated

treatment) or tamoxifen alone (designated control) and monitored for impact on tumour growth.

**Results** – In the phase 2 study gefitinib treatment was well tolerated with an overall clinical benefit rate of 33.3% (n=18/54). Pre-treatment oestrogen receptor positivity was associated with tumour response to gefitinib (p=0.015), longer TTP (p = 0.015), and with clinical benefit (CB) in 53.6 % of the ER+ acquired tamoxifen resistant patients. In contrast, the clinical benefit rate was minimal in the steroid receptor negative patient cohort (11.5%). All patients in this series expressed detectable levels of EGFR, but high pre-treatment levels of EGFR predicted a poorer outcome (p=0.075) Only patients achieving CB had a significant fall in Ki67 staining as measured at 8 weeks versus pre-treatment levels (p=0.024), and that Ki67 levels were lower in CB than PD patients at this time. We observed lower levels of EGFR phosphorylation at this time point in some CB patients. Further examination of the CB pts who showed a >10% decline in EGFR phosphorylation revealed decreases in phosphorylation of MAPK and also in Ki67.

TAMR xenografts expressed high levels of EGFR as previously observed in vitro. Their growth was significantly inhibited by gefitinib (p=0.039) over the study period while after only 2 weeks of gefitinib treatment tumours showed a decrease in the level of Ki67 staining (p = 0.068).

**Conclusion** –Acquired tamoxifen resistance in vivo both in patients and in a xenograft model appears to be in part mediated through EGFR pathway signalling and this can be blocked and growth inhibited with gefitinib. In ER

negative tumours the effects of gefitinib were less striking, suggesting alternative signalling pathways are dominant in promoting their growth despite obvious overexpression of EGFR.

## **2 Published and Presented Work**

### **2.1 Papers**

1. Wyld, L., Gutteridge, E., Pinder, S.E., James J.J., Chan, S.Y., Cheung, K.L., Robertson, J.F.R. and Evans, A.J. “Prognostic Factors for Patients with Hepatic Metastases from Breast Cancer” British Journal of Cancer 2003 Jul 21; 89: 284-90

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factor receptor signalling and oestrogen receptor activity in clinical breast cancer.” Endocrine Related Cancer. 2005 Jul; 12 Suppl 1:S99-S111.

7. Gutteridge E., Agrawal A., Nicholson R.I., Cheung K.L., Robertson J.F., Gee J.M. “The Effects of Gefitinib in Tamoxifen Resistant and Hormone Insensitive Breast Cancer: A Phase II Study” Accepted by the International Journal of Cancer September 2009

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## **2.2 Abstracts**

1. Wyld, L., Gutteridge, E., Pinder, S., James, J.J., Chan, S.Y., Cheung, K.L., Robertson, J.F.R. and Evans, A. “Liver Metastases from Breast Cancer: Prognostic factors” European Journal of Surgical Oncology (2002) 28: 762

2. Evans, A., James, J., Cornford, E., Chan, S., Burrell, H., Pinder, S., Gutteridge, E., Robertson, J., Hornbuckle, J., and Cheung, K.L. “Brain Metastases from breast cancer: identification of a high risk group” European Journal of Cancer 2003 Vol 1 (supplement 4):26

3. James, J.J., Evans, A.J., Pinder, S.E., Gutteridge, E., Cheung, K.L., Chan, S. and Robertson, J.F. “Bone metastases from breast carcinoma: histopathological – radiological correlations and prognostic features” European Journal of Cancer 2003 Vol 1 (supplement 4):16

4. Gutteridge, E., Evans, A., James, J., Chan, S., Robertson, J.F. and Cheung, K.L. “Parenchymal and Pleural Metastases in Breast Cancer – Prognostic

Features” European Journal of Surgical Oncology 2003 Vol 29 (Number 9):827

5. Robertson, J.F.R., Gutteridge, E., Cheung, K.L., Owers, R., Koehler, M., Hamilton, L. “A Phase II Study of ZD1839 (Iressa) in tamoxifen-resistant ER-positive and endocrine-insensitive (ER-negative) breast cancer” Breast Cancer Research and Treatment 2002 Vol 76 (supplement 1): 96

6. Gutteridge, E., Cheung, K.L., Owers, R., Koehler, M., Hamilton, L., Gee, J., Nicholson, R.I. and Robertson, J.F.R. “Gefitinib (Iressa, ZD1839) has activity in patients with oestrogen receptor (ER) negative breast cancer and ER positive breast cancer that has acquired resistance to Tamoxifen: results from a phase II study” European Journal of Cancer 2003 Vol 1 (supplement 4):11

7. Gee JM, Gutteridge E, Robertson JF, Wakeling AE, Jones HE, Nicholson RI. “Biological markers during early treatment of tamoxifen resistant breast cancer with gefitinib.” British Journal of Surgery 2005 Vol 92 (Supplement 1):13

### **2.3 Oral Presentations**

1. Wyld, L., Gutteridge, E., Pinder, S., James, J.J., Chan, S.Y., Cheung, K.L., Robertson, J.F.R. and Evans, A. “Liver Metastases from Breast Cancer: Prognostic factors” British Association of Surgical Oncologists Annual Scientific Conference November 2002

2. Evans, A., James, J., Cornford, E., Chan, S., Burrell, H., Pinder, S., Gutteridge, E., Robertson, J., Hornbuckle, J., and Cheung, K.L. “Brain Metastases from breast cancer: identification of a high risk group” The 8<sup>th</sup> Nottingham International Breast Cancer Conference

3. James, J.J., Evans, A.J., Pinder, S.E., Gutteridge, E., Cheung, K.L., Chan, S. and Robertson, J.F. “Bone metastases from breast carcinoma: histopathological – radiological correlations and prognostic features” The 8<sup>th</sup> Nottingham International Breast Cancer Conference
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5. Gutteridge, E., Cheung, K.L., Owers, R., Koechler, M., Hamilton, L., Gee, J., Nicholson, R.I. and Robertson, J.F.R. “Gefitinib (Iressa, ZD1839) has activity in patients with oestrogen receptor (ER) negative breast cancer and ER positive breast cancer that has acquired resistance to Tamoxifen: results from a phase II study” Presentation in the Patey Prize session at The Society of Academic and Research Society, Belfast 2004
6. Agrawal A, Mustafa T, Gutteridge E, Robertson JFR, Cheung KL “Response to further endocrine therapies following de novo resistance to first line endocrine therapy for advanced breast cancer (ABC)” The 10<sup>th</sup> Annual Nottingham Breast Cancer Conference, Nottingham 2005
7. Gee JM, Gutteridge E, Robertson JF, Wakeling AE, Jones HE, Nicholson RI. “Biological markers during early treatment of tamoxifen resistant breast cancer with gefitinib.” The Association of Surgeons Great Britain and Ireland, Glasgow 2005



## **2.4 Posters**

1. Gutteridge, E., Evans, A., James, J., Chan, S., Robertson, J.F. and Cheung, K.L. “Parenchymal and Pleural Metastases in Breast Cancer – Prognostic Features” The 8<sup>th</sup> Nottingham International Breast Cancer Conference 2003 and at the British Association of Surgical Oncologists Annual Scientific Conference November 2003
2. Robertson, J.F.R., Gutteridge, E., Cheung, K.L., Owers, R., Koehler, M., Hamilton, L. “A Phase II Study of ZD1839 (Iressa) in tamoxifen-resistant ER-positive and endocrine-insensitive (ER-negative) breast cancer” The 25<sup>th</sup> Annual San Antonio Breast Cancer Conference December 2002
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4. Robertson JFR, Gutteridge E, Cheung KL, Wakeling A, Gee J Nicholson RI “Clinical Efficacy of Gefitinib, Iressa, in Tamoxifen-Resistant ER Positive and in ER Negative Breast Cancer” The 28<sup>th</sup> Annual San Antonio Breast Cancer Conference December 2004
5. Gee JM, Gutteridge E, Robertson JF, Wakeling AE, Jones HE, Nicholson RI. “Biological markers during early treatment of tamoxifen resistant breast cancer with gefitinib.” The 28<sup>th</sup> Annual San Antonio Breast Cancer Conference December 2004
6. Agrawal A, Gutteridge E, Cheung KL, Hyman-Taylor P, Wakeling A, Robertson JFR..“Efficacy and tolerability of gefitinib in oestrogen receptor negative and tamoxifen resistant oestrogen receptor positive locally advanced

or metastatic breast cancer.” The 29<sup>th</sup> Annual San Antonio Breast Cancer  
Conference December 2005

### **3 Declaration and Acknowledgements**

I declare that this thesis has been composed by me, and that the work described was performed by me or by myself in conjunction with others. I identified, recruited and obtained consent from the patients for the study. The vast majority of the clinical assessment and tumour biopsies were performed by me in the outpatient setting. I performed all of the word processing, statistical analysis and graph generation using University licensed software packages – Microsoft Word and SPSS version 17. I would like to thank Professor J.F. Robertson and the Tenovus group particularly Professor R.I. Nicholson and Dr Julia Gee for the hypothesis behind the study. All tumour sectioning, immunostaining and immunocytochemical assessment was carried out at the Tenovus Institute, University of Cardiff, Wales. I would like to acknowledge Julia Gee, Pauline Findley, Sue Kyme and Lynne Farrow for the time and effort spent in the processing and analysis of the samples. The in vivo mouse xenograft work was undertaken at the University of Nottingham. I would like to acknowledge Professor Sue Watson, Teresa Morris and Andrew Mackenzie for their contributions.

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This work has not been presented at any previous application for a degree by others or myself.

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## **4 Introduction**

### ***4.1 Background***

Cancer is a class of diseases characterized by an imbalance between cell division and apoptosis. The natural history of cancers is to spread either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis. Metastatic spread can occur via the bloodstream or the lymphatics.

Damage to the cell's DNA leads to mutations in the genes coding for proteins. This can give rise to uncontrolled cell division and disruption of the normal regulation of cellular growth. Several stepwise mutations may be required for such a malignant transformation and these mutations can be precipitated by carcinogens (i.e. radiation, chemicals or physical agents) or can occur spontaneously.

Cancer is not a new disease. It is mentioned in ancient writings from India, Egypt and Greece. In Greek mythology, Karkinos was a crab that came to the aid of the Lernaean Hydra as it battled Heracles. Karkinos bit Heracles in the foot, but was crushed beneath his heel. Hippocrates was born in 460 BC and was one of the first to characterize benign and malignant tumours in the breast. He introduced the term “karkinos” and our word carcinoma is derived from this.

## ***4.2 Epidemiology***

Breast cancer is now the most common cancer in the UK. Each year more than 44,000 women and 300 men are diagnosed with breast cancer [1]. Breast cancer rates have increased by more than 50% over the last twenty years, with the highest rates in Anglia and Oxford, North Thames and the South West. In England the NHS breast screening programme picks up around 14,000 cases of breast cancer each year. Eighty percent of all breast cancers are diagnosed in women aged 50 and over. Around 430,000 women are diagnosed with breast cancer in the European Union every year [2]. Worldwide, more than a million women are diagnosed with breast cancer annually. The highest rates of breast cancer occur in Northern Europe and North America and the lowest rates are in parts of Africa and Asia [3]. However even in the latter regions the incidence of breast cancer is rising.

Breast cancer is now the second most common cause of death from cancer in women after lung. Each year in the UK more than 12,000 women and around 100 men die from breast cancer with around 1,400 deaths from breast cancer in women under 50. However more than half of breast cancer deaths are women aged over 70. Since peaking in the late 1980s breast cancer death rates have fallen by a third. This is in part due to the National Health Service breast screening programme which is thought to save approximately 1,400 lives each year, and in part due to advances in adjuvant chemo-endocrine therapies [4].

The incidence of breast cancer worldwide varies dramatically according to geography, environmental and individual risk factors [5]. The main risk factors for breast cancer can be classified as:



### **4.2.1 Advancing Age**

Age is by far the strongest risk factor of a woman for developing breast cancer. A woman in her early 20s has a 1:15,000 risk, this then increases to 1:50 by age 50 and to 1:10 by age 80. This equates to an overall lifetime risk of 1:9 [6].

### **4.2.2 Genetic Factors**

A woman with one affected first degree relative (mother or sister) has approximately double the risk of breast cancer of a woman with no family history of the disease; if two (or more) relatives are affected, her risk increases further [7, 8]. Many women have concerns regarding the genetic factors leading to the development of breast cancer however less than 5% of breast cancers are thought to be genetic in origin [9]. One study conducted in the Family History clinic setting revealed that a woman's perception of life time risk is difficult to quantify and often inaccurate. This risk assessment improves with counselling but many women overestimate their chances of developing the disease [10]. Small proportions of women have a strong family history of breast cancer, calculated by various established risk models, and are at very high risk. Mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 account for the majority of families with four or more affected members and 2-5% of all breast cancers. These genes have a 50-80% penetrance. Hence carriers have a 50-80% chance of developing the disease at some point in their lifetime, although the majority of this risk is passed by the time a person reaches 50 years of age [11].

### **4.2.3 Reproductive / hormonal factors**

Women in developed countries have higher breast cancer rates vs. the underdeveloped world. This is in part due to the fact that women in developed countries tend to have fewer children and breastfeed less. Childbearing at a younger age is protective with a relative risk reduction of 7% for each birth, as is the number of pregnancies even in the absence of breastfeeding [12]. Breastfeeding further decreases relative risk, by 4% per year, which means that a woman must breastfeed for a year to reduce her risk by this amount [13]. Early age at menarche is a significant risk factor as is late onset of menopause. This is due to the length of time of exposure to oestrogens. Postmenopausal women have a lower breast cancer risk than age matched premenopausal controls [14].

Exposure to endogenous and exogenous oestrogen in the form of hormone replacement therapy (HRT) or the oral contraceptive pill has been shown to be a factor in the development of breast cancer. The current use of oral contraceptives slightly increases the risk of breast cancer (1.24:1), but there is no significant excess risk ten or more years after stopping use (1.01:1) [15]. HRT use increases the risk of breast cancer and reduces the sensitivity of mammography due to its effects on breast tissue density. The breasts remain dense so making it difficult to detect small cancers [16]. The risk of breast cancer for current users of HRT is 66% higher than for women never exposed.

After the menopause, when ovarian function ceases, body fat in the form of adipose tissue is the primary source of endogenous oestrogen, hence overweight and obese women are exposed to higher levels of oestrogen. About

8% of breast cancer cases in the UK may be attributable to obesity [17]. A systematic review by the International Agency for Cancer Research (IARC) found that obesity was associated with several forms of cancer, including breast cancer (IARC 2002).

#### **4.2.4 Mammographic Density**

Mammographic density is related to the risk of breast cancer. Women with denser breasts have a 2-6 times increase in their relative risk of breast cancer compared to women who do not have dense breasts [18]. Several studies have shown that the proportion of radiographically opaque tissue seen on mammogram is important in assessing breast cancer risk [19].

#### **4.2.5 Proliferative Breast Pathology**

Breast tissue hyperplasia represents a field change within the breast tissue. Women who have had biopsies that showed proliferative breast disease without atypia have a 2-fold increased relative risk, while women with atypical hyperplasia have a 2-5 fold increased relative risk of breast cancer [20, 21]. The presence of lobular carcinoma in situ (LCIS) increases the risk of developing cancer in either breast whereas ductal carcinoma in situ (DCIS) may progress to invasive cancer within the affected breast.

### ***4.3 Natural History of the Disease***

The natural history of the disease following formation of the primary tumour is to locally invade and to metastasize leading to the death of the patient from carcinomatosis.

Breast cancer is a heterogeneous disease often with a long course. One of the first documented studies of its natural history took place at the Middlesex Hospital, involving 250 patients between 1805 and 1933 [22]. These patients presented, in the main, with advanced disease (74% had metastatic cancer, 23% were locally advanced and only 2% were primary cancers), no treatment was given to the patients although medical records were documented. The onset of disease was estimated with good accuracy. Records showed that 39% of patients presented within 1 year of onset of first symptom and only 7% presented within 6 months of onset. Despite no intervention, the median survival was 2.7 years with a 5 year survival of 18% and a 10 year survival of 4%.

A subsequent review compared breast cancer mortality rates in surgically treated patients [23]. It concluded that for patients presenting with advanced disease, breast cancer mortality had remained unchanged for 40 years with up to 40% having a fatal outcome despite intervention. Now the mainstay of early breast cancer treatment is surgery. Randomized trials comparing surgical treatments of breast cancer showed that radical mastectomy offered no benefit over simple mastectomy followed by radiotherapy [24]. Furthermore, in terms of survival, radical mastectomy was no better than quadrantectomy followed by radiotherapy for early breast cancers [25]. This has had an impact in the development of surgical techniques aiming to provide safe oncological clearance whilst achieving a good cosmetic result.

## **4.4 Classification of Breast Cancer**

### **4.4.1 Primary Breast Cancer**

Early primary breast cancer (PBC) is defined as a mass of <5cm, with no clinical fixed axillary nodal involvement, and no invasion of the skin or underlying tissues. Primary local surgical options are based upon tumour size. Patients with tumours larger than 3-4 cm (depending on breast size) or with evidence of multifocal disease are advised against breast conservation. Completion mastectomy after wide local excision is recommended if the specimen margins are involved and further excision is thought unlikely to attain clear margins or would compromise cosmesis such that breast reconstruction would provide a better cosmetic result. Outside of a clinical trial intact breast irradiation routinely follows breast conservation surgery otherwise the rate of local recurrence is unacceptably increased. Radiotherapy is given to mastectomy flaps if the tumour is grade 3 and the lymph nodes are involved &/or there is vascular invasion. If the axilla is known to be involved pre-operatively (diagnosed on clinical or ultrasound guided core biopsy of suspicious nodes) then patients undergo an axillary clearance. If the axilla is clear then a standard four node sample is carried out or sentinel node biopsy is performed, the latter to locate and specifically sample the first draining node. Histological evidence of axillary metastases in the sampled women leads to a full axillary clearance or axillary irradiation [26].

#### **4.4.2 Elderly Primary Breast Cancer**

For the purposes of this thesis elderly primary cancer (EPC) is as above in a patient over the age of 70. Primary tamoxifen or surgery have been shown to be comparable in terms of metastasis and overall survival yet the high local failure rate on primary endocrine therapy suggests that optimal management of the fit elderly should include surgery [27]. However there remain situations where co-morbidities preclude surgical treatment for a patients' breast cancer and in this setting pharmacological agents may be used with careful monitoring.

#### **4.4.3 Locally Advanced Primary Breast Cancer**

Locally advanced breast cancer (LAPC) encompasses a wide spectrum of disease with differing behaviours and responses to therapy. Locally advanced primary cancer was first formally classified by Haagensen, in the 1940s and is defined as a mass of greater than 5cm in size, or a mass of any size with invasion of or fixity to skin or underlying tissues. He identified that patients with such features have a poor prognosis following surgery.

Since the use of screening mammography has become widespread, the proportion of patients who have locally advanced disease at diagnosis has decreased. Data from the United States National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program, indicates that 7% of patients are locally advanced at diagnosis. Cancers can become locally advanced due to neglect, aggressive disease or site i.e. male breast cancer. According to the SEER data, the 3- and 5-year relative survival rates for

women with stage III breast cancer are 70% and 55%, respectively. Median survival for women with stage III disease is 4.9 years [28].

The standard treatments are multimodality therapy (i.e. surgery, radiotherapy (neo) adjuvant chemotherapy &/or endocrine therapy) or alternatively in the oestrogen receptor positive group the option of primary endocrine therapy (PET) with other treatments sequentially as required. Local treatment alone is associated with high rate of local recurrence. The poor survival is due to the presence of occult metastases at time of diagnosis. Over time there has been a change of emphasis towards combining local and systemic treatments to target both loco-regional disease and distant metastases. A trial in our unit was designed to answer the question “Does up-front multi-modal treatment confer an advantage over single systemic treatment?” The two arms compared initial hormone treatment versus a combination of neo-adjuvant chemotherapy, radical mastectomy, radiotherapy and adjuvant hormone therapy. It concluded that the probability of developing distant metastases similar in both arms with no significant difference in survival [29]. This has been further supported recently by the long-term outcome of a series of almost 200 patients treated by PET which showed a 5 year breast cancer specific survival of over 80% (Mathews et al Presented at BASO meeting Nov 2007). Therefore the management of locally advanced breast cancer in our unit routinely includes PET and these women may be managed within a trial setting.

#### **4.4.4 Advanced Breast Cancer**

Advanced or metastatic cancer (ABC) has been defined as involvement of supraclavicular nodes, contra lateral axillary nodes or distant sites i.e. bone, lung, liver, soft tissue or brain. More recently it has been suggested that

ipsilateral lymph node involvement should be returned to primary operable breast cancer. Metastatic breast cancer is an incurable disease and systemic treatments are given to try and prolong life while at the same time trying to palliate symptoms. Survival rates for breast cancer have been improving for more than 20 years. The estimated relative five-year survival rate for women diagnosed in England and Wales in 2001-2003 was 80%, compared with only 52% for women diagnosed in 1971-1975 as reported by the Office for National Statistics. The estimated relative twenty year survival rate for women with breast cancer has gone from 44% in the early 1990s to 64% for the most recent period.

Although the majority of women with breast cancer are successfully cured by their surgical procedures and adjuvant therapies, between 30- 40% will develop metastatic disease [30]. Even in patients with small node negative breast cancers, and an excellent short term prognosis, a number will eventually develop distant metastases and die of the disease [31].

Once breast cancer is disseminated it can be controlled but not cured. At the time of writing there is no agent or combination of agents that can control the growth of breast cancer cells indefinitely. The development of resistance to therapeutic agents is thus inevitable and poses a significant problem for clinicians and patients. New agents in the treatment of breast cancer are trialled initially in the advanced setting before becoming accepted adjuvant therapies. The 2008 NICE draft guidelines for the treatment of oestrogen receptor positive metastatic breast cancer recommend endocrine therapy unless there is a high disease load (where chemotherapy would be appropriate to achieve early control). In post-menopausal women an aromatase inhibitor should be used as



first line endocrine therapy (or second line if the disease has progressed on tamoxifen). In pre-menopausal women tamoxifen is still the first line treatment of choice with ovarian suppression offered on tamoxifen failure.

## ***4.5 Prognostic Factors of the Primary Tumour***

### **4.5.1 Nottingham Prognostic Index (NPI)**

In 1982 Haybittle and coworkers [32] reported a series of 387 patients with primary breast cancer from Nottingham. Using a multiple-regression analysis of prognostic factors and survival, a prognostic index was created. The index was based on lymph-node stage, tumour size and pathological grade and was expressed as a formula:

$$\text{NPI} = (0.2 \times \text{tumour size in cm}) + \text{grade (1 to 3)} + \text{nodal stage (1 to 3)}$$

This index allowed patients to be classified into groups according to their prognosis (Figure 1):

1. Good prognostic group 1 – 3.4, 5 year mortality rate 3%
2. Moderate prognostic group 3.4 – 5.4, 5 year mortality rate 7%
3. Poor prognostic group 5.4 and above, 5 year mortality 30%

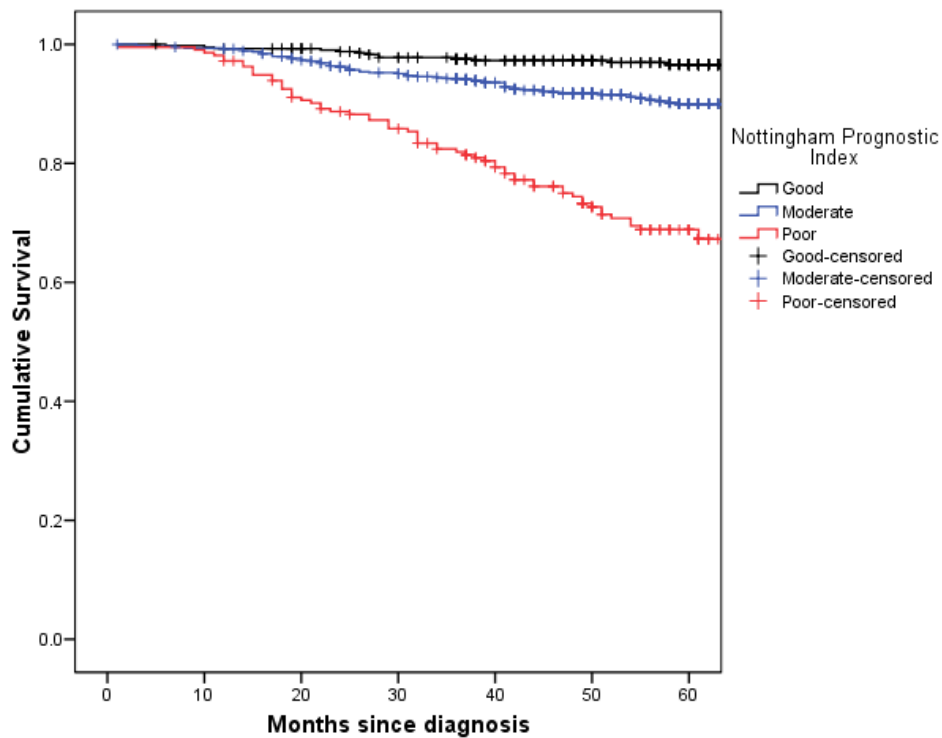


Figure 1: Nottingham Prognostic Index 1

The index was validated by recalculating for the same patients with over 5 years further follow-up and also applied prospectively to a further group of 320 patients.[33] Patients are now divided into 5 prognostic groups (Figure 2):

1. Excellent (<2.4),
2. Good (2.41-3.4),
3. Moderate I (3.41-4.4),
4. Moderate II (4.41-5.39)
5. Poor (>5.4).

Patients in the excellent group have an almost normal survival and comprise 11% of those assessed, a further 10% have a very poor prognosis. The index is used to categorise patients to allow appropriate counselling and to tailor adjuvant therapies to those felt to derive the most clinical benefit.

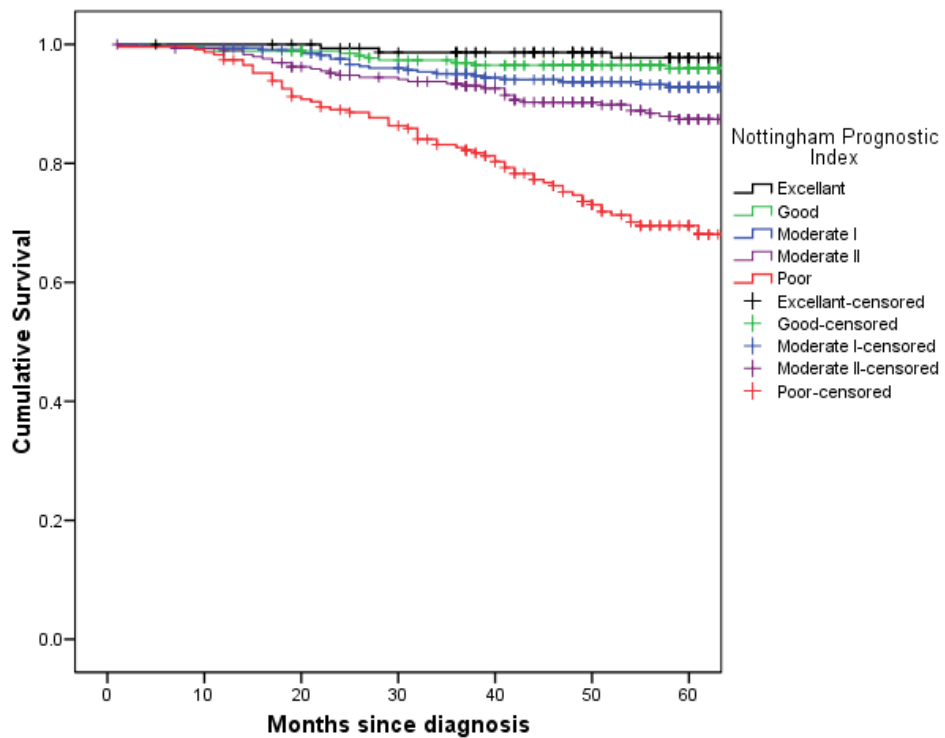


Figure 2: Nottingham Prognostic Index 2

#### 4.6 Endocrine Sensitivity

In the 1896 a Scottish surgeon by the name of George Beatson reported that the ovaries might have some influence on the behaviour of breast cancers [34]. This work was based upon observations in sheep that the ovaries controlled lactation and thus as he put it ‘one organ held sway over another’. In 1895 he performed a bilateral salpingo-oophrectomy in a 33-year-old lady who had an extensive inoperable local recurrence of her previously surgically treated breast cancer. After several months her breast cancer had completely regressed, a state now called complete response. Encouraged by this he employed the same technique on another of his patients with advanced breast cancer. In this case a marked but temporary regression was seen, a partial response. In the first two cases therefore Beatson identified what has since become known as endocrine

sensitivity with subsequent acquired resistance. This was the first observation of its kind and the mechanisms involved are still not fully understood.

This work caused other doctors and scientists to look at this phenomenon in more detail. Boyd in 1900 analysed a series of 54 women who had had oophrectomies for advanced breast cancer. He found a 35% response rate, with varying levels of cancer regression, within this group. In 1905 Letts reported a 30% response rate in advanced breast cancer treated by surgical oophrectomy. Surgical or radiation oophrectomy gradually became accepted as a treatment for advanced breast cancer however response rates remained low. There were attempts to perform bilateral adrenalectomies [35] and even pituitary ablation [36].

#### ***4.7 Adjuvant Therapies***

Systemic adjuvant therapies for primary operable breast cancer are given with the intention to eradicate any occult metastatic disease and to prevent recurrence, thus prolonging disease free survival and overall survival. They include hormonal and cytotoxic agents and for a long period of time tamoxifen was the antioestrogen of choice. Adjuvant systemic therapies are routinely offered to women who fall into intermediate and high risk groups. For women in the low risk group it is felt that the risks and benefits are more closely balanced and these are discussed with each individual patient. Recent NICE (National Institute of Clinical Excellence) guidelines have recommended the following adjuvant protocols for oestrogen receptor positive post-menopausal women [37].

1. Primary use of aromatase inhibitor for 5 years after surgery
2. Switch to aromatase inhibitor after 2 or 3 years of tamoxifen, to continue for a total of 5 years
3. Extended endocrine therapy with an aromatase inhibitor for 3 years after completing 5 years of tamoxifen.

For pre-menopausal women with early breast cancer the recommendations (according to the draft NICE guidelines 2008) are that tamoxifen should continue to be used as there is evidence that aromatase inhibitors may be ineffective in this group. Adjuvant ovarian ablation is not recommended for those receiving tamoxifen and chemotherapy. Ovarian ablation should be offered for those who choose not to have chemotherapy where recommended.

The object of ovarian ablation by whatever means is to deprive the breast cancer cells of circulating oestrogen. It can be achieved by pharmacological means i.e. chemotherapy or LHRH analogues or by ovarian irradiation or oophrectomy. In premenopausal women the primary source of oestrogen synthesis is via the ovaries. In the postmenopausal women oestradiol is synthesized from testosterone and androstendione in the liver and body fat. The reaction is catalyzed by the aromatase enzyme. Pharmacological compounds such as tamoxifen target oestrogen receptor binding whereas anastrozole, letrozole and exemestane target aromatase enzyme activity. Fulvestrant is a selective oestrogen receptor downregulator and, due to its lack of endometrial stimulation and absence of cross resistance with tamoxifen, is currently being evaluated.

#### **4.7.1 Tamoxifen**

Tamoxifen is a selective oestrogen receptor modulator. Tamoxifen was introduced to clinical practice in the 1970s [38]. Initially trialled in the advanced setting [39, 40] it was found to be as effective as high dose oestrogens or androgens in postmenopausal women and was well tolerated. It was licensed for use in the United Kingdom in 1973 and it is presently the drug of choice in the adjuvant setting for premenopausal women alongside ovarian ablation in oestrogen receptor positive breast cancer.

Tamoxifen is given as a once daily oral dose of 20mg. It takes approximately 4 weeks for steady state plasma levels to be reached at this dose. It is largely metabolized by the liver and can cause derangement of liver enzymes particularly the transaminases. Tamoxifen and its metabolites are antioestrogens which act by competitively binding with the oestrogen receptor thus blocking oestradiol from binding with the oestrogen binding site. This prevents the formation of the oestrogen – oestrogen receptor complex which blocks the activation of downstream growth transcription genes leading to increased apoptosis and decreased cell proliferation by blocking the cell cycle in G1 [41, 42]. However at low doses tamoxifen has oestrogen agonist properties, this is thought to explain the “flare effect” sometimes seen when treatment is commenced. This can be detected in symptoms such as increased pain in patients with bone metastases, or biochemical abnormalities, such as a transient increase in the blood tumour markers (CEA and CA15-3) [43].

Tamoxifen has been extensively trialled and The Early Breast Cancer Trialists’ Collaborative Group performed a meta-analysis of all randomised trials that

began before 1990 [44]. The objective was to compare the effects of tamoxifen vs. placebo and to determine the appropriate duration of treatment. In total, there were 37 000 cases from 55 trials. There were 8000 known oestrogen receptor negative cases, 18 000 known oestrogen receptor positives and 12 000 with an unknown status. For those patients oestrogen receptor positive or unknown, there was a 21%, 29%, and 47% proportional reduction in recurrence for patients treated with 1 year, 2 years and 5 years of tamoxifen respectively this was associated with a reduction in mortality at these time-points of 12%, 17% and 26%. . There was also a reduction in the incidence of contralateral breast cancers. . A more recent analysis by the same group has shown that the absolute 15-year gain in disease free survival after 5 years of tamoxifen compared to placebo in oestrogen receptor positive patients was 11.8% with 9.2% gain in breast cancer specific mortality [45]

Another recent meta-analysis confirmed that five years of adjuvant tamoxifen significantly reduced recurrences (41%) and mortality (31%) within this group with a 34% reduction in distant recurrences. The analysis revealed the overall breast cancer recurrence risk to be approximately 3% per year and confirmed a higher rate of recurrence in node positive vs. node negative patients (4% vs. 2%) [46]. In these large series there was an observed reduction in contralateral breast cancer which raised the possibility of using tamoxifen as a hormonal preventative agent. From 1992 – 1997 13 388 high risk women (as calculated by the modified Gail model) were enrolled in The National Surgical Adjuvant Breast and Bowel Project (NSABP) P-1: Breast Cancer Prevention Trial (BCPT) [47]. The incidence of oestrogen receptor positive invasive breast cancers was reduced by almost a half and there was a similar reduction in non-

invasive cancers. The International Breast Cancer Intervention Study 1 (IBIS-1) [48] confirmed the positive outcomes from the NSABP P-1 trial. This multinational study recruited 7152 women with an increased risk of breast cancer over a 10 year period. The incidence of oestrogen receptor positive invasive cancer was reduced by 32% following 20mg tamoxifen for 5 years, with no effect on the incidence of oestrogen receptor negative tumours. This risk reduction has been confirmed in a large Italian National Trial which showed a reduction of 82% in the highest risk group [49].

The main minor side effects of tamoxifen therapy described were gastrointestinal upset, weight gain, hot flushes and vaginal dryness [50]. However larger studies showed that the agonist properties of tamoxifen can lead to more serious adverse events such as thromboembolic events and endometrial carcinoma [51, 52]. Tamoxifen also has an agonistic effect on the bone profile.

#### **4.7.2 The Aromatase Inhibitors**

Initially aromatase inhibitors such as the first generation aminoglutethimide and the second generation formestane, fadrozole (Afema) and rogletimide were only used as second line therapeutic agents due to their significant toxicities. This changed with the development of third generation aromatase inhibitors such as anastrozole which proved to be superior to other second line agents in both tolerability and efficacy [53]. As existing second line agents had efficacies similar to tamoxifen [54] several studies were established to compare tamoxifen with the new aromatase inhibitors in the advanced setting. In 2001 the preliminary results from the International Letrozole Breast Cancer Group provided convincing evidence that letrozole as first line therapy was superior to



tamoxifen in terms of efficacy and tolerability in postmenopausal advanced breast cancer [55]. These findings were confirmed following an update in 2003 where the time to progression was 9.4 months in the letrozole group versus 6.0 months in the tamoxifen group, with an objective response rate of 32% versus 21% in favour of letrozole [56].

Having established the superiority of aromatase inhibitors in the advanced disease setting, several studies were instigated to investigate their role in early breast cancer.

#### **4.7.2.1      Anastrozole (Arimidex)**

Anastrozole is a third generation non steroidal aromatase inhibitor. It reversibly inhibits the aromatase enzyme, responsible for the conversion of androgens into oestrogenic metabolites. In postmenopausal women oestrogen is generated from the adrenal glands and body fat by the action of the aromatase enzyme. Thus blocking this enzyme leads to a decrease in the circulating levels of oestrogen. In premenopausal women, where the main source of oestrogen is the ovaries, aromatase inhibitors lead to a small decrease in oestrogen which activates the hypothalamus and pituitary axis to increase gonadotropin secretion, which in turn stimulates the ovary to increase androgen production, counter-acting the effects of the drug. Anastrozole was first described in 1994 and is given as a once daily oral dosage of 1mg [57]. An overview of two phase III trials demonstrated that anastrozole, at doses of 1 and 10 mg once daily, was well tolerated and as effective as megestrol acetate in the treatment of postmenopausal women with advanced breast cancer who progressed following tamoxifen treatment [53]. A dose comparison analysis recommended a daily dose of 1mg as this showed comparative efficacy with

the larger dose [58]. Following on from this anastrozole (1mg) was compared with tamoxifen (20mg) as first line therapy for post menopausal women with advanced breast cancer. A total of 668 patients (340 in the anastrozole arm and 328 in the tamoxifen arm) were randomized to treatment and followed-up for a median of 19 months. The median time to progression, objective response rate and clinical benefit rate was similar for both treatments demonstrating that anastrozole was at least equivalent to tamoxifen. There was a lower observed incidence of thromboembolic events and vaginal bleeding in the anastrozole group. The authors concluded that anastrozole should be considered as first-line therapy for postmenopausal women with metastatic breast cancer [59]. In addition a small neoadjuvant pre-surgical study in oestrogen receptor positive postmenopausal patients with large cancers demonstrated a median reduction in tumour volume of 75% with anastrozole after 12 weeks of treatment, leading to an increased incidence of suitability for breast conserving surgery vs. pretreatment [60].

As a result of these promising findings the Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial was instigated to investigate anastrozole and tamoxifen as monotherapies or in combination as 5 years of adjuvant therapy in 9,366 post-menopausal women with early breast cancer. After a median follow-up of 68 months, anastrozole significantly prolonged disease-free survival (575 events with anastrozole vs. 651 with tamoxifen), time-to-recurrence (402 events with anastrozole vs. 498 with tamoxifen), and significantly reduced distant metastases (324 with anastrozole vs. 375 with tamoxifen) and contralateral breast cancers (35 with anastrozole vs. 59 with tamoxifen). Anastrozole was also associated with fewer side-effects than

tamoxifen, especially gynaecological problems and vascular events, but arthralgia and fractures were increased. The authors concluded that anastrozole should be the preferred initial treatment for postmenopausal women with localised hormone-receptor-positive breast cancer [61].

The Arimidex-Nolvadex 95 (ARNO 95) and Austrian Breast and Colorectal Cancer Study Group 8 (ABCSG 8) trials investigated anastrozole in the sequential adjuvant setting. 3224 post-menopausal women with early stage hormone receptor positive breast cancer were randomised to either continue tamoxifen or switch to anastrozole after two years of adjuvant tamoxifen therapy. The combined analysis of these two randomised, open-label trials was recently published after a median follow up of 28 months. Those in the anastrozole arm had a 40% decrease in the risk of loco/regional or distant recurrence as compared with the tamoxifen group (67 events with anastrozole versus 110 with tamoxifen) supporting a switch to anastrozole after 2 years of tamoxifen therapy. Anastrozole therapy was significantly associated with an increased risk of bone fractures and as such a baseline bone density scan is recommended prior to commencing aromatase inhibitors [62].

#### **4.7.2.2      Letrozole (Femara)**

Letrozole is a specific reversible non-steroidal aromatase inhibitor. It has shown superior efficacy to tamoxifen in large multi-centre clinical trials. The BIG 1-98 trial has recruited 8028 post-menopausal women with hormone receptor positive breast cancer to examine the use of tamoxifen or letrozole as monotherapy for 5 years or a sequence of either 2-3 years of tamoxifen or letrozole followed by 2-3 years of the other. The primary analysis in December 2005 up to the point of treatment switch (median follow up: 25.8 months)

demonstrated an 18% relative risk reduction in disease-free survival in the letrozole arm when compared to tamoxifen. Letrozole monotherapy reduced the risk of recurrence with five year disease free survival rates of 84% versus 81.4% in the tamoxifen group. In addition there was a highly significant 30% reduction in distant recurrence with letrozole [63, 64]. With such a short follow up period it is not entirely surprising that there was no significant difference in overall survival between the two groups but data from the sequential arms, due to be published this year should provide more information and enable us to decide on the benefit of and order of sequencing.

The MA.17 study was a randomised, double-blind, placebo-controlled trial conducted by the National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG). The hypothesis was that extended adjuvant therapy with letrozole could effectively address the risk of late recurrence and hence affect overall survival. From August 1998 to September 2002, 5187 postmenopausal women (46% of whom were node positive) were randomised to take a total of 5 years of tamoxifen followed by what was intended to be 5 years of placebo versus 5 years of letrozole. After the first interim analysis and a median follow up of only 2.4 years the trial was unblinded due to the obvious superiority of letrozole versus placebo with regard to disease free survival in the sequential regime. [65]. There were 207 local, contralateral or metastatic recurrences, 75 being in the letrozole and 132 in the placebo group. No statistical difference in overall survival was observed. In the final data analysis, after 30 months of follow-up, there was a significant 40% reduction in the risk of distant metastasis in the letrozole group compared with placebo and the estimated four-year disease free survival was significantly higher in women who received

letrozole. This letrozole effect was irrespective of nodal status and prior chemotherapy and was the first trial to report a survival advantage with an aromatase inhibitor in early post-menopausal breast cancer [66].

#### **4.7.2.3 Exemestane (Aromasin)**

Exemestane differs from anastrozole and letrozole in that it is a steroidal aromatase inhibitor which forms a permanent bond with the aromatase enzyme complex. There is evidence that a switch to exemestane after 2-3 years of adjuvant tamoxifen conveys an advantage. The Intergroup Exemestane Study (BIG 97-02 trial) recruited 4742 post-menopausal women with early breast cancer and who were disease free after two to three years of adjuvant tamoxifen. The primary aim was to assess whether 2-3 years of tamoxifen followed by 2-3 years of exemestane, with a total sequential time-span of 5 years, would improve disease-free survival when compared to tamoxifen alone. Patients were randomised to continue tamoxifen treatment or switch to exemestane for completion of five years of adjuvant therapy and the analysis was on an intention to treat basis [67, 68]. At a median of 30.6 months of follow up an interim analysis was performed that revealed that the sequential arm had an improved outcome with a 32% risk reduction and absolute benefit of disease-free survival of 4.7%. At this point overall survival was similar in both arms. A further analysis was performed at a median follow up of 55.7 months revealing an 18% improvement in time to distant recurrence in the oestrogen receptor positive / oestrogen receptor unknown group with a switch to exemestane. They were also able to demonstrate a 17% improvement in overall survival in this group compared with standard adjuvant tamoxifen therapy.

#### **4.7.2.4 Fulvestrant (Faslodex)**

Fulvestrant is an entirely different class of endocrine agent; it is a selective oestrogen receptor modulator that causes dose dependent degradation of the oestrogen receptor. There is growing interest in fulvestrant due to its lack of endometrial stimulation and absence of cross resistance with tamoxifen. Two phase III trials (0020 and 0021) have shown that it is at least as efficacious as anastrozole as a second line hormonal agent in advanced disease [69, 70]. In these studies a total of 851 postmenopausal women with locally advanced or metastatic breast carcinoma were randomised to receive either a monthly intramuscular injection of 250mg fulvestrant or oral 1mg anastrozole daily. These patients had failed first line treatment (mostly with tamoxifen) and were hormone receptor positive. Only those who had a measurable or assessable disease, with a life expectancy > 3 months, were included. After a median follow up of 27.0 months, 319 (74.5%) patients on fulvestrant and 322 (76.1%) on anastrozole had died. Median survival was comparable at 27.4 and 27.7 months respectively and the authors concluded that fulvestrant was as efficacious as anastrozole in the second line treatment of metastatic breast cancer [71].

### **4.8 Metastatic Breast Cancer**

#### **4.8.1 Presentation**

Patients with metastatic breast cancer can present in a variety of ways. They may complain of pain, lethargy or fatigue. They may be anaemic or have deranged liver biochemistry. They may present with breathlessness or hypercalcaemia. A study in the 1970s reported that symptoms are a reliable

indicator of relapse with 57.6% of patients presenting symptomatically. A further 32.1% were detected by self/physician examination [72]. Once metastatic disease is suspected the standard investigations include a CT of thorax and upper abdomen and a bone scan. Blood samples should be analysed for full blood count, urea and electrolytes, liver function tests and calcium. In addition one or other of the blood tumour markers CEA and CA15-3 will be elevated in up to 80% of patients with metastatic disease [73].

A study in the early 1970s assessed the initial sites of metastatic presentation in 145 patients [74]. The most common first site of distant spread was bone (51%), followed by lung (17%), brain (16%), and liver (6%). The remaining 10% of patients had multiple metastatic sites. The overall median survival time after metastasis was 12 months for bone and lung lesions, three months for brain lesions, and only one month for liver metastasis. The median survival of patients with multiple metastatic sites was 7.5 months. They noted a longer time to metastasis in node negative patients and a shorter survival with metastases in those patients who had initially been lymph node positive.

A large population based study in the Netherlands looked at patterns of disease and survival over two separate time periods in 868 patients. In the time period 1985-1994 314 patients were analysed. The sites of disease were documented (bone 47%, liver 15%, lung/pleura 12%, brain 3%, skin 9% and unknown 22%) the median survival over this time period was 17 months. A second group of patients, who presented in the time period 1995-2002, were included in the study (n=554). These patients had a higher incidence of bone (55%), liver (23%) and lung/pleural metastases (18%). The distribution in the other disease sites was not significantly different. The authors surmised that this increase in

may be due to differences in detection rather than a true reflection of changing disease distribution. The median survival in this later group was significantly prolonged at 20 months [75].

In another small series data was collected on a consecutive series of 100 patients presenting with metastatic breast cancer [76]. Skeletal metastases comprised the majority, with 67% of patients having skeletal involvement. Liver ultrasound examination showed metastatic disease in 32% of patients. Chest radiographs demonstrated metastatic disease in 42% of patients.

Between July 1997 and December 2001, 492 patients presented to the Nottingham City Hospital with metastatic breast carcinoma [77]. Of these, 267 patients had bone metastases at initial presentation with metastatic disease of whom, 34% of patients had bone as their only metastatic site. Sites of first presentation of metastatic disease were prospectively recorded, as were histological features of the primary tumour (tumour type, histological grade, lymph node stage, tumour size and oestrogen receptor (ER) status). The radiological features of the bone metastases, the metastasis-free interval and blood serum tumour marker levels (CEA, CA15-3) at presentation with metastases were all recorded. There was a significant association between the development of bone metastases and lower grade tumours ( $p=0.019$ ), ER-positive tumours ( $p<0.0001$ ) and the lymph node stage of the primary tumour ( $p=0.047$ ). A multivariate analysis found that metastasis-free interval, additional sites of metastatic disease other than bone, ER status and serological tumour marker levels all independently contributed to survival from time of presentation with bone metastases.



## **4.8.2 Prognostic factors**

The prognosis with metastatic breast cancer is very much dependant on disease site although this in turn is significantly associated with biological features of the tumour (e.g. hormone receptor status and histological grade). A retrospective study of 439 women with recurrent breast cancer from a single institution reported a median survival of 24 months and five-year overall survival of 18%. Using a univariate analysis, pathological tumor size at diagnosis, nodal status at diagnosis, negative hormone receptor, adjuvant chemotherapy, short disease free interval, location of recurrence and number of metastatic sites, were significantly associated with shorter survival from first relapse. In the multivariate analysis, only the site of recurrence, axillary lymph node status at diagnosis, ER status and DFI remained independently associated with decreased survival after first relapse [78].

Patients with soft tissue or bone metastases fare much better than those with visceral involvement [79]. Isolated soft tissue metastases have a median survival of 50 months [80].

### **4.8.2.1 Liver Metastases**

Review of the literature reveals that liver metastases are found in 6–25% of patients [80, 81]. A study reviewing 312 patients with liver metastases reported that the primary tumours were commonly poorly differentiated [82] and the median survival was 3.8 months. At presentation 60% of patients had hepatomegaly, 13% were jaundiced and 7% had ascites. The presence of jaundice ( $P < 0.001$ ), ascites ( $P = 0.01$ ) or hepatomegaly ( $P = 0.01$ ) were all associated with a particularly poor prognosis. While any degree of elevation of

bilirubin ( $P$  less than 0.001) or alkaline phosphatase ( $P = 0.003$ ) conferred a poorer prognosis.

In a study in our unit analyzing 145 patients with liver metastases a median survival of 4.23 months was seen (range 0.16 to 51 months) with a 27.6% 1-year survival. Factors that significantly predicted a poor prognosis on univariate analysis included symptomatic liver disease ( $p < 0.001$ ), deranged liver function tests, the presence of ascites ( $p < 0.003$ ), histological grade 3 disease at primary presentation, advanced age, oestrogen receptor (ER) negative tumours, CEA of over 1000 ng ml<sup>-1</sup> and multiple liver metastases. Multivariate analysis of pre-treatment variables identified a low albumin, advanced age and ER negativity as independent predictors of poor survival [83].

Survival may be prolonged by chemotherapy or endocrine therapy and a small proportion of patients may survive for 5 years (3%) or even 10 years (1%) with these therapies [84]. Current recommendations are that patients with asymptomatic, oestrogen receptor (ER) positive liver metastases may be treated with endocrine therapy [85]. Those with symptomatic metastases or ER negative tumours are treated with combination chemotherapy such as FEC (5-fluorouracil, epirubicin and cyclophosphamide) or CMF (cyclophosphamide, methotrexate, 5-fluorouracil) [84, 86]. There is some evidence that performing surgical resection of hepatic metastases in carefully selected patients can lead to significant increases in survival with low operative morbidity and mortality raising the possibility of incorporating surgery into our management of these patients [87, 88].

#### **4.8.2.2 Lung Metastases**

Isolated lung metastases have been reported to occur in 10-20% of all women with breast carcinoma [89]. A study on lung metastases [90] reviewed 249 patients presenting to our unit between October 1997 and January 2003 with pleural or parenchymal metastases at initial presentation. Survival from metastatic diagnosis was compared with prognostic features of the primary cancer at presentation, patient characteristics, disease extent and tumour markers (CEA and CA15-3) at the time of metastatic presentation. Median survival was 14.2 months (range 2 weeks – 15 years). The histological subtype, nodal stage, presence of vascular invasion and Nottingham Prognostic Index of the primary had no effect on survival. A multivariate analysis revealed ER negativity and the presence of other visceral metastases as independent predictors of poor outcome. Elevation of the tumour markers at presentation was not an indication of poor prognosis.

In selected cases a resection may increase patient survival. A study of 467 patients [91] revealed that in 84% a complete metastatic resection was possible. They were able to achieve 5 year survival rates of 38% with low operative morbidity and mortality. Prognostic factors were a disease-free interval of > or = 36 months and the presence of solitary lung metastases.

#### **4.8.2.3 Cerebral Metastases**

Cerebral metastases, although an uncommon initial presentation of metastatic breast cancer, often occur later in the disease process and confer a dismal prognosis with a reported median survival of only 4 months [92]. The central nervous system appears to be a “sanctuary site” and is not accessible by

conventional systemic therapies due to the function of the blood brain barrier. A recent study from the Nottingham Breast group attempted to identify a subgroup of women at high risk of brain metastases [93]. The radiological reports of 219 women presenting with metastases aged less than 70 years who had subsequently died were examined. The type, frequency, temporal occurrence and survival with brain metastases were documented. Correlations were sought between the frequency of brain metastases and age at metastatic presentation, tumour grade, histological type and oestrogen receptor (ER) status. Of the 219 women, 49 (22%) developed brain metastases. The development of brain metastases was related to young age ( $p = 0.0002$ ), with 43% of women under 40 years developing brain metastases. Brain metastases were more common in women whose tumours were ER negative (38%) compared with women with ER-positive disease (14%) ( $p = 0.0003$ ). A group of women were identified (age under 50 years and ER negative) with a 53% risk of developing brain metastases. This group included many women who had chemotherapy for visceral metastases, and 68% had either stable disease or disease response at other sites at the time of brain metastases presentation. This subgroup of women at high risk may benefit from pre-emptive medical intervention, such as screening or prophylactic treatment.[9]. Another study analysed patients with presenting with cerebral metastases from breast cancer over a 20 year period and found that 17% were considered suitable for surgical resection followed by whole brain radiotherapy whilst the remainder had radiotherapy plus/minus systemic chemotherapy. In this series the median overall survival was 6.1 months (range 0.4-82.2 months). Eight patients survived for at least 2 years after their diagnosis of brain metastases and all of

these had surgical resection and/or chemotherapy in addition to radiotherapy.

In patients who simply had palliative radiotherapy there was a significant improvement in symptoms. They concluded that selected patients could have significantly improved survival with a more aggressive approach [94].

#### **4.8.2.4 Histology of the Primary Tumour**

The primary tumour type has bearing on survival with metastatic disease.

The metastatic pattern at presentation and the prognosis with metastases of 48 patients with carcinomas with tubular features (45 tubular mixed and three pure tubular) and 302 patients with tumours of ductal of no special type (DNST) were compared. A retrospective study from a prospectively maintained database of all patients who developed metastatic disease from carcinoma of the breast in Nottingham, U.K., since 1997, was performed. We recorded site of first presentation with metastatic disease, radiological features, histological features and characteristics of the primary tumour. The group of patients with tubular features were older at metastatic presentation (63.9 years vs. 59.6 years;  $p=0.012$ ), had a longer disease-free interval (87 months vs. 34 months;  $p<0.001$ ) and a longer survival with metastases ( $p<0.002$ ). This group were less likely to have liver metastases (23% vs. 41%;  $p=0.028$ ), in particular multiple liver metastases (50% vs. 71%;  $p=0.015$ ) than the patients with DNST. Other factors known to be associated with prolonged survival, such as low histological grade of the primary invasive tumour and positive oestrogen receptor (ER) status, were more common in the group of patients with tumours with tubular features (Grade 1: 33% vs. 3%; Grade 2: 42% vs. 25%; Grade 3: 25% vs. 72%;  $p<0.001$ ), (ER positivity 76% vs. 52%;  $p=0.009$ ). When patients

with grade 2 tumours were compared, the age at metastatic presentation, disease-free interval and the presence of multiple liver metastases were still significantly different between the two groups. Patients with metastatic breast carcinoma with tubular features have a longer survival with metastases than patients with metastatic DNST carcinoma. This improved survival can be explained by better well-recognised prognostic features, such as metastatic site pattern, histological grade, ER status and disease-free interval [95].

#### **4.8.2.5 Response to Treatment**

The median survival of patients with metastatic breast cancer is between 2 – 3 years from the time of symptomatic presentation. Survival can be prolonged in those who respond to systemic therapy and long-term responders are almost invariably patients who have endocrine sensitive tumours. While the rate of response is important in evaluating an endocrine treatment, the duration of response should be at least 6 months. Any short-lived response of a lesser duration seldom translates into a survival advantage. Over the years we have most commonly used the UICC criteria to classify response [96].

1. Complete response (CR) – complete disappearance of lesion
1. Partial response (PR) –  $> 50\%$  reduction of bi-dimensional product
2. Stable disease (SD) –  $\pm 25\%$  increase of bi-dimensional product
3. Progressive disease (PD) –  $> 25\%$  increase of bi-dimensional product or appearance of a new lesion

It is worth noting that not all lesions are measurable and/or assessable.

Another method of assessing tumour response to treatment is by the RECIST criteria (Response Evaluation Criteria in Solid Tumours). The criteria were

published in February, 2000 by an international collaboration including the EORTC, NCI and the NCIC encompassing Europe, the United States and Canada. They are slightly different from the UICC criteria [97] and have since been adopted as the standard in the majority of clinical trials.

- Complete Response (CR) - disappearance of all target lesions
- Partial Response (PR) -  $> 30\%$  reduction of bidirectional product
- Stable Disease (SD) - Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD,
- Progressive Disease (PD) -  $> 20\%$  increase of bidirectional product or the appearance of a new lesion

When looking at response rates, patients should be grouped as having progression (PD) and clinical benefit (CR + PR + SD) rather than responders (CR + PR) and non-responders (SD + PD). Patients who have achieved an SD at 6 months have the same survival as those who have CR or PR at 6 months. It is only the group of patients with PD at 6 months who will have a survival disadvantage. This finding has been confirmed for first, second, and third line endocrine therapies [98-100].

## **5 Factors Inducing Breast Cancer Growth**

There are two main classes of breast cancer growth receptors. The oestrogen and progesterone receptors are steroid hormone receptors. The other main family is the growth factor receptors including the insulin-like growth factor receptor and the epidermal growth factor receptor family. These receptors exist in inactive or activated states within the cell. Activation or phosphorylation is the addition of a phosphate ( $\text{PO}_4$ ) group to a protein molecule or a small molecule. In 1906, Phoebus A. Levene at the Rockefeller Institute for Medical Research identified phosphate in the protein Vitellin (phosvitin) [101]. However, it took another 20 years before Eugene P. Kennedy described the first ‘enzymatic phosphorylation of proteins [102]’. Reversible phosphorylation of proteins utilises enzymes called kinases and is an important regulatory mechanism which occurs within cells. Phosphorylation results in a conformational change in the structure in many receptors, causing them to become activated and is designated in this thesis as a “p”.

### ***5.1 Steroid Hormone Receptors***

#### **5.1.1 The Oestrogen Receptor**

The oestrogen receptor (ER) is involved in normal breast development and is found in a higher concentration in oestrogen receptor positive breast cancer cells than in the surrounding normal tissue [103, 104]. Two isoforms of oestrogen receptor have been identified, ER alpha and ER beta, both have the same binding capacity with oestradiol but they have different actions in the regulation of gene expression [105]. For the purposes of this thesis we will concentrate on ER alpha. The oestrogen receptor was first described in 1976. It



is a 66kDa oestrogen binding protein that is expressed on the nucleus in 50 – 80% of breast cancers. [104, 106, 107] It is responsible for transmitting oestrogenic growth signals from outside a cell into its nucleus. It has both an oestradiol binding domain and a DNA binding domain [104]. Lipophilic oestradiol enters the cell's nucleus and binds with the oestrogen receptor. This causes the release of heat shock protein and allows dimerization to occur. The receptor – hormone complex can then bind to the oestrogen response element (ERE) on the DNA. This leads to gene transcription and increased expression of proteins encoded by these genes. These genes include the progesterone receptor [106]. These genes, via growth factors, establish growth stimulatory effects. The action of the oestrogen receptor can be regulated by other receptors. The activation of growth factor receptor pathways, such as epidermal growth factor receptor and insulin-like growth factor receptor can have direct or indirect effects upon oestrogen receptor transcription [108]. Phosphorylation of 2 sites within the hormone independent AF-1 region of oestrogen receptor alpha (Ser167 and Ser118), has been shown to mediate anti oestrogen resistance by promoting oestrogen independent growth. Ser167 activation is induced by AKT and Ser118 is activated by the MAPKinase pathway [109]. In ER positive patients phosphorylation of Ser167 has been associated with low tumour grade, lymph node negativity, relapse-free and overall survival [110, 111]. Its expression also predicts for response to endocrine therapy in metastatic cancer and significantly longer survival after relapse [112]. Conversely Ser118 activation is correlated with HER2 expression and lack of endocrine response.

The National Surgical Adjuvant Breast and Bowel Project, instigated in 1977, reported that increased disease free survival was associated with increasing levels of both oestrogen and progesterone receptor within the tumours of women with primary operable breast cancer and positive axillary nodes [111]. Oestrogen receptor positivity remains today the best indicator of response to endocrine manipulation.

### **5.1.2 The Progesterone Receptor**

The presence of a progesterone receptor (PgR) is regarded as evidence of a functioning oestrogen receptor. The progesterone receptor binds with DNA in much the same way as the oestrogen receptor causing activation of gene transcription. In addition it recruits co activator molecules to further increase transcription. There is evidence that it can also cause rapid changes in intracellular signalling via the MAP kinase pathway [113]. PgR has been confirmed as an independent predictor of endocrine responsiveness in a study of 342 patients [114]. In this prospective study from the Southwest Oncology Group sub-classification of patients by ER and PgR showed a non-significant trend between response and increasing levels of PgR in the low expressing ER group. No such trend was seen in the high level of ER group. The authors were able to conclude that knowledge of PgR would allow improved assessment of patient prognosis. This seems to be particularly relevant in the elderly population [115].

Another analysis [116] classified 99 patients for the four possible phenotypes of ER and PgR combination ( ER positive/ PgR positive, ER positive/PgR negative, ER negative/PgR positive and ER negative/PgR negative.) 67% of

double receptor positive tumours showed responsive or static disease compared to 25% of double receptor negative tumours. Tumours of mixed phenotype showed an intermediate response rate of 46%. They concluded that identification of double positive and double negative tumours might give a better estimate of response, but that the choice of therapy would not be influenced.

A large study of clinical outcomes in patients from two large databases analyzed their steroid receptor status [117]. The first database contained 3,739 patients who did not receive any systemic adjuvant therapy and 1,688 patients who received adjuvant endocrine therapy alone. The second database contained 10,444 patients who received adjuvant endocrine therapy alone. Biochemical ER and PgR assays were identically performed in two different central laboratories. The authors found that in univariate and multivariate analyses, the prognostic significance of PgR status among systemically untreated patients is uncertain. However in the systemic adjuvant endocrine treatment group, multivariate analyses, including lymph-node involvement, tumour size, and age, revealed that a positive PgR status is independently associated with disease-free and overall survival. For recurrence, the reduction in relative risk was 25% for ER-positive/PgR-negative patients and 53% for double positive patients, compared with double negative patients. Patients with ER-positive/PgR-negative tumours had a reduction in relative risk of death of 30% to 38%, compared with patients with double negative tumours. For ER-positive/PgR-positive tumours, the reduction of the risk of death was between 46% and 58%, indicating that double positive patients obtain more benefit from endocrine therapy. More recent studies into PgR function have suggested that

ER-positive/PgR-negative cancers are more likely to be driven via growth factor signalling pathways and may be more likely to respond to EGFR TKIs [118].

## **5.2 The Growth Factor Receptors**

### **5.2.1 Epidermal Growth Factor Receptor**

The epidermal growth factor receptor (EGFR) is a 170kDa type I tyrosine kinase receptor that is expressed in 40% to 60% of human breast cancers [119, 120]. It was first discovered in 1978 [121]. It is also known as HER1 and is part of a family of tyrosine kinases including HER2, HER3 and HER4. It has a cysteine rich extracellular binding domain and an intracellular cytoplasmic tyrosine kinase domain. The antibody used to detect EGFR (Clone 111.6, Neomarkers) blocks the binding of EGF to the extracellular domain of the EGFR receptor. Its ligands also include transforming growth factor alpha and heparin-binding EGF-like growth factor. Ligand binding to the extra cellular domain leads to homo and heterodimerisation with HER2, HER3 or HER4 which triggers autophosphorylation via intrinsic intracellular protein-tyrosine kinase activity at the Y992, Y1045, Y1068, Y1148 and Y1173 residues in the C-terminal domain [122] This phosphorylation ultimately results in cellular proliferation, angiogenesis and prolonged cell survival via downstream signalling pathways such as the MAPKinase pathway. The antibody used to detect phosphorylated (p) EGFR (Tyr 1173, Chemicon) recognizes the major autophosphorylation site of the human EGF receptor Y113 without interacting with the non-phosphorylated EGF receptor or with other unrelated phosphotyrosine proteins. There is evidence that EGFR is expressed and over-expressed in a wide range of human solid cancers including breast cancer.

EGFR over-expression occurs in epithelial-derived tumours, such as non-small cell lung, colon, breast, prostate and head and neck cancers [123, 124]. Expression of EGFR has been correlated with poor prognosis in primary and recurrent breast cancer [125, 126]. HER2 is over expressed in approximately 20% of breast cancers and the two receptors are co-expressed in 10 - 30%. In tumours with overexpression of EGFR there was an 87% chance of increased HER2 expression and conversely tumours with high levels of HER2 had a 35% chance of overexpressing EGFR [127].

This co-expression is associated with a poorer prognosis than either receptor alone and may be due to synergy between these genes leading to a sustained and independent proliferation of breast cancer cells [128]. The expression of EGFR is inversely correlated with oestrogen receptor expression and this has been widely studied and reported. The majority of these groups have also reported a negative association with the progesterone receptor [129, 130]. In particular patients who are ER negative and EGFR positive have a poorer prognosis [131]. Cell culture studies have shown that tamoxifen sensitive MCF7 cell lines grown up in the presence of tamoxifen express EGFR at the development of resistance [132]. Furthermore the combination of ER and EGFR blockage leads to a much decreased breast cancer cellular growth rate in vitro [133].

### **5.2.2 HER2**

HER2 oncoprotein is a 185 KDa protein known by a variety of names, (c-neu, and cerbB-2). It has been shown to be an independent predictor of poor prognosis when phosphorylated or when co expressed with EGFR [127] and is associated with high-grade tumours [134]. Belonging to the same family of

receptors as EGFR, HER2 is present in normal breast tissue but is overexpressed in approximately 20% to 30% of human primary breast cancers [135, 136]. This overexpression causes shortening of the G1 phase of the cell cycle and early S phase entry, which leads to hyper proliferation. When phosphorylated HER2 heterodimerises with EGFR [137], HER 3 [138] and HER 4 it activates a downstream phosphorylation cascade which includes the intracellular cytoplasmic MAPKinase signalling pathway [139-141].

A soluble fragment of the HER2 oncogene product can be detected in patients with primary breast cancer. Serum levels were also elevated at the time of recurrence [142]. An association between HER2 oncoprotein serum levels in breast cancer patients and poor prognosis has also been identified [143].

### **5.2.3 Insulin-like Growth Factor Receptor**

The insulin growth factors are essential for normal development and are produced by breast stromal cells, acting as paracrine factors for cell growth [144]. The insulin like growth factor receptor (IGFR) is expressed widely in breast cancer. It is a transmembrane tyrosine kinase receptor but structurally different from EGFR. Its ligands are the insulin like growth factors IGF1 and IGF2. They have been shown to be potent mitogens in cell culture [145]. Activation of the IGFR leads to autophosphorylation [146] and phosphorylation of a signalling protein IRS-1. IRS-1 stimulates the MAPKinase pathway leading to mitogenesis, enhanced growth properties and reduced apoptosis. IGFR mRNA has been detected in primary breast tumours and in the serum of patients of affected patients and its expression correlates

with ER status [147]. It has been implicated in the regulation of the progesterone receptor [148, 149] and cfos gene expression [150]. IGF1 and oestradiol work synergistically to promote breast cancer cell growth, oestrogen causing increased responsiveness to the proliferative effects of the IGFs [147, 151]. Increased IGFR levels in breast cancer are associated with early recurrence of the tumour at the primary site [152] and significantly shortened median survival [153]. Cell culture studies have shown that treatment with tamoxifen leads to down regulation of IGF1 phosphorylation of the IGFR and inhibition of IRS-1 (insulin receptor substrate 1) signalling [154, 155] and that disruption of IGFR signalling leads to suppression of metastatic disease and increased survival [156, 157]. At the development of tamoxifen resistance IGFR signalling is increased, interacting with EGFR [158]. Resistance to gefitinib in tamoxifen resistant cell culture lines is associated with increased IGFR signalling in the face of EGFR receptor blockade [159].

#### **5.2.4 Mitogen Activated Protein Kinase**

There are three major mitogen activated protein kinase (MAPK) pathways active in human tissues: the extra cellular-signal regulated kinases (ERK1/ERK2), the c-jun N-terminal kinases (JNK), and p38 kinase. They play an essential role in transmitting and amplifying signals which result in cell proliferation and differentiation as well as cell death. Activation of the Ras/MAP kinase (ERK1/ERK2) pathway by insulin or insulin-like growth factor is important in receptor tyrosine kinase-induced signal transduction, including HER2 and IGFR-mediated signalling and so is particularly relevant in breast cancer [160, 161]. The activated form of MEK, one of the components of the MAP Kinase pathway has been detected in high levels in

human breast cancers [162]. Ras is a low molecular weight G protein which when bound to GTP activates a serine theonine kinase (Raf). This then phosphorylates and activates the extra cellular signal related protein kinases 1 and 2 (ERK1/2) [163]. The duration of MAPKinase activity determines the cellular response. The downstream effectors of ERKs are nuclear transcription factors such as c-myc, cfos, and c-jun, which trigger cell proliferation via direct action on gene expression. These have been shown to be activated and expressed in the nucleus of human breast cancer specimens [164] The MAPKinase p38 pathway is thought to be involved in cell adhesion and in cell culture, activation of this pathway leads to increased adhesion to a collagen matrix [165] and contributes to metastatic potential [166]. Long term oestrogen deprived cell lines express elevated levels of activated MAPKinase and use these pathways for cell proliferation in the absence of oestrogen as a growth promoter [166-168]. Blockage of the MAPKinase signalling pathway leads to decreased tumour cell growth in vitro [169].

### **5.3 Downstream Effectors**

#### **5.3.1 AKT**

AKT is a serine/theonine kinase protein and is a downstream effector of HER2, IGFR and EGFR [170]. It is a major regulator of growth factor mediated cell cycle progression and increased cell survival via reduced apoptosis.[171-173]. Three isoforms have been identified, AKT-1, -2, and 3 and these are routinely expressed in both normal and malignant breast tissue [174]. Oestrogen leads to activation of AKT via the PI3K (phosphoinositide-3-kinase) pathway but has also been shown to activate AKT via a receptor independent path in oestrogen receptor negative breast cancer cell lines [175]. Cell lines which have



developed tamoxifen resistance have high levels of activated AKT-1 [176]. Endocrine responsive tumours expressing activated AKT have a higher incidence of distant metastases [177] and a shorter overall survival [109], a lower response to adjuvant endocrine therapy and a higher rate of local recurrence following radiotherapy [178]. Co-expression of HER2 and activated AKT confers a particularly poor prognosis [179, 180]. High levels of AKT predict for recurrence in node negative breast cancers [181]. In addition activated AKT levels are associated with multidrug resistance in cell lines [182]. Small studies in metastatic patients have confirmed that the expression of activated AKT and HER2 is an indicator of poor response to endocrine therapy in this setting [183].

Treatment with tamoxifen leads paradoxically to an increase in activated AKT levels [184] via an agonist effect and blockage of AKT activity with an n-3 fatty acid (EPA) restored tamoxifen sensitivity [185]. Recently studies have shown that anti-oestrogen resistant cell lines have no overall increase in AKT but have elevated activated AKT levels. The cells responsiveness to antioestrogens can be restored by AKT inhibitors [186], this implicates AKT in the development of tamoxifen resistance.

### **5.3.2 bcl2**

The expression of the bcl2 proto-oncogene coding for a mitochondrial protein is associated with prolonged cell survival and prevention of programmed cell death [187]. bcl2 expression is strongly correlated with ER and PgR expression and inversely correlated with EGFR and Ki67 expression [188, 189]. bcl2 is an inhibitor of apoptosis and is overexpressed in more than half of all human cancers [190]. Over expression of bcl2 occurs in 40% to 80% of human breast

tumours. bcl2 is not an independent prognostic marker in breast cancer patients, in part because most bcl2 positive breast cancers express ER and/or PgR. This positive association of bcl2 with hormone receptors in breast cancer may explain its apparent correlation with response to hormone therapy. However, diminished apoptotic response caused by bcl2 over expression is associated with cellular resistance to chemotherapeutic drugs [191] and has been implicated in the development of metastases [192, 193] In oestrogen receptor-positive MCF7 breast cancer cells tamoxifen induced time and concentration dependent down regulation of bcl2 at both the mRNA and protein level. This down-regulation of bcl2 correlated with tamoxifen-induced apoptosis [194] and was significantly associated with quality of response to tamoxifen in vivo [195].

### **5.3.3 cfos**

Fos protein is the product of the oestrogen related nuclear transcription factor cfos, a proto-oncogene. cfos can be induced by both steroid hormones and peptide growth factors and is involved in many signalling pathways. cfos expression is associated with a failure to respond to endocrine therapy. Sustained elevated levels of cfos expression were significantly associated with further factors, notably peptide growth factors and their receptors (e.g., EGFR, TGF alpha), as well as with the proliferation marker Ki67. cfos is found at lower levels in those tumours expressing markers of endocrine responsiveness (e.g., oestrogen receptor, and also ER-mediated markers i.e., PgR, bcl2) [196]. Steroid hormones and antioestrogens affect fos protein expression via their actions on the cfos oestrogen response element (ERE). As a constituent of the AP-1 complex, the fos protein initiates a cascade of events that result in

increased proliferation, prolonged cell survival and decreased differentiation. Increased transcription for genes coding for cell metastasis and invasion is seen with elevated levels of cfos expression [197] as is endocrine resistance. Elevated cfos, proliferation and increased cellularity are also seen at the time development of tamoxifen resistance [198].

## **5.4 Proliferative Indices**

### **5.4.1 Ki67**

Ki67 is a monoclonal antibody which recognizes a nuclear antigen expressed by cells in G1, S, G2, and M phases of the cell cycle but not Go. Ki67 is a proliferative index marker [199]. A high level of Ki67 expression is associated with highly proliferative tumours and endocrine insensitivity [200]. A significant decrease in Ki67 levels has been shown to correlate with endocrine response [201] and recurrence free survival [202].

High Ki67 expression is associated with ER negativity, EGFR positivity and decreased patient survival [203-206]. It has been shown that expression of genes related to apoptosis and cell death i.e. bcl2 are down regulated in tumours that have high levels of Ki67 expression [207].

Short term pre-surgical studies have demonstrated decreases in Ki67 expression with a variety of endocrine agents. In one of the first neoadjuvant pre-surgical studies tamoxifen was given to 21 primary breast cancer patients and cytology examined after 2 weeks. There was an observed decrease in oestrogen receptor and Ki67 expression which correlated with tumour response [208]. Similar effects have been reported in oestrogen receptor positive post-menopausal patients receiving tamoxifen as primary endocrine therapy [195].

A further study demonstrated falls in Ki67 levels but no relationship with recurrence over a reasonably short period of follow up [209]. Vorozole, a nonsteroidal aromatase inhibitor was first trialled in metastatic breast disease in 1994 where it showed efficacy comparable to megestrol acetate and high dose oestrogens [210]. In a randomised trial with tamoxifen, given for 12 weeks, in post-menopausal women awaiting surgery, there was a greater reduction in Ki67 with vorozole vs. tamoxifen and this correlated with tumour response [211].

More recently the IMPACT neoadjuvant study (n=330) has reported a greater reduction in Ki67 staining in oestrogen receptor positive post-menopausal primary breast tumours with anastrozole vs. tamoxifen vs. the combination. They also reported that the Ki67 decreases were greater in the high steroid receptor positive groups. There was also an increase in Ki67 levels in HER2 negative vs. HER2 positive patients (significant in the tamoxifen only group at 2 weeks and the anastrozole only group at 12 weeks.)([212].

Newer agents such as fulvestrant are also able to decrease Ki67 expression. Once again in post-menopausal oestrogen receptor positive pre-surgical studies a dose dependent reduction in oestrogen receptor, progesterone receptor and Ki67 staining has been demonstrated [213].

Gefitinib has been investigated in two recent phase II, randomised neoadjuvant studies. One stipulated that the patients were oestrogen receptor positive and EGFR positive and investigated gefitinib daily vs. anastrozole in combination with gefitinib. There were very large decreases in Ki67 staining in both groups with a greater decrease seen in the combination group [214]. Another larger study investigated 206 oestrogen receptor positive postmenopausal patients and

randomised them to 3 groups. The intent was to administer anastrozole alone, anastrozole for 2 weeks followed by the addition of gefitinib or the upfront combination. Once again there was a significant decrease in Ki67 staining of approximately 80% at 16 weeks for both anastrozole and gefitinib but interestingly in this case no benefit to the combination. The authors noted that within the progesterone receptor positive cohort there was a statistically significant detrimental effect on response for the combination regime [215].

## **5.5 Possible Mechanisms for the Development of Tamoxifen Resistance**

Resistance to endocrine manipulation can occur on initial treatment, designated “de novo” resistance or after a period of response, “acquired resistance”. The mechanisms of resistance to tamoxifen therapy have been widely studied and provide the backbone of our understanding of the phenomenon of hormone resistance. A significant number of patients will go on to benefit sequentially from other methods of oestrogen blockade. There is no doubt that the mechanisms involved are extremely complex and involve interaction between the oestrogen driven pathways and the growth factor receptor pathways.

### **5.5.1 Loss of Oestrogen Receptor**

Cells which are de novo resistant to tamoxifen do not express the oestrogen receptor. It has been suggested that oestrogen receptor expression falls at the development of tamoxifen resistance [216] [217], however this is in association with maintained oestrogen receptor function. In pre-surgical studies tamoxifen has been shown to decrease levels of oestrogen receptor expression after short periods of treatment [208, 218] and this oestrogen receptor decrease is associated with tumour response [195].

### **5.5.2 Oestrogen Receptor Isoforms**

Two isoforms of the oestrogen receptor exist and are designated alpha and beta. ER beta was first reported to be expressed in rat, human and mouse tissues. Its role in breast cancer is not yet fully understood. A splice variant (ER beta cx) has been identified which has no affinity for oestradiol and which, if co-expressed with ER alpha, acts as a negative repressor, effectively

blocking its function [219]. Both isoforms can exist within cells and can cross-signal with each other. Conflicting studies have suggested either that tumours co-expressing ER alpha and ER beta tend to be node positive with a trend for a more aggressive phenotype [220], or that ER beta expression is associated with an absence of lymph node metastases and low tumour grade [221] and increased survival [222]. The significance and application of routine ER beta testing is unclear at the present time [105].

### **5.5.3 Mutation of Oestrogen Receptor**

The expression of the oestrogen receptor within human breast cancer is heterogeneous. Mutant oestrogen receptor mRNA has been detected in many breast cancers and cell lines [223]. Variants can be created in a variety of ways including single or multiple exon deletions, deleted or truncated transcripts, insertions or point mutations. All these mutations lead to the coding of different proteins ultimately leading to potential differences in oestrogen receptor function. For instance high expression of the clone 4 truncated ER mRNA was found in tumours with poor prognosis and endocrine resistance [224] and a further study identified a deletion of exon 5 to be associated with hormone resistance [225]. ER beta has a mutation creating ER beta cx which represses the function of ER alpha as discussed in the previous section [105].

### **5.5.4 Phosphorylation of the Oestrogen Receptor**

The oestrogen receptor can be phosphorylated by oestradiol leading to activation of downstream growth factor pathways leading to increased transcription. This growth promoting cycle can be blocked by the administration of tamoxifen. However there are certain phosphorylation sites

on the oestrogen receptor that can be activated by growth factor receptors and downstream effectors leading to a positive feedback loop. This provides an escape mechanism for the continued growth of oestrogen receptor positive breast cancer cells in the face of oestrogen blockade. The Ser167 and Ser118 sites within the hormone independent AF-1 region of oestrogen receptor alpha have been shown in part to mediate tamoxifen resistance. Ser167 is mediated by AKT and Ser118 by the MAPKinase pathway [109]. In addition the transcription factors cfos and jun can bind directly to the activating protein 1 site providing an alternative docking site for the oestrogen receptor [226] leading to increased transcription.

#### **5.5.5 Altered Cellular Levels of Tamoxifen**

Reduced tumour levels of tamoxifen in the face of satisfactory serum concentrations have been detected in vivo. Tamoxifen resistant (TAMR) ER-positive MCF7 human breast cancer xenografts had markedly lower intracellular tamoxifen levels vs. their tamoxifen sensitive counterparts. They exhibited isomerization of the potent antioestrogenic metabolite trans-4-hydroxy-tamoxifen to the less potent cis isomer. Metabolic tolerance, as manifested by alterations in cellular concentrations of tamoxifen and its metabolites, may thus be one mechanism for acquired tamoxifen resistance in breast cancer [227]. A further study found lower tumour than serum levels of tamoxifen in patients with recurrent tumours [228]. This is possibly due to active secretion of the drug from the cancer cell. Another possible mechanism is the reduced uptake of tamoxifen by the cancer cell.



### **5.5.6 Metabolism of Tamoxifen**

Tamoxifen is metabolised in the liver via the cytochrome p450 2D6 to its active metabolites which have a much greater affinity for the oestrogen receptor. Patients with variant forms of this gene may not receive full benefit from tamoxifen because of slow metabolism of the tamoxifen prodrug into its active metabolite 4-hydroxytamoxifen [229] and there has been some debate regarding gene testing prior to trial recruitment (ABS at BASO 2009). The two main metabolites are N-desmethyltamoxifen and 4-hydroxytamoxifen. N-desmethyltamoxifen is detected mostly in the serum. 4-hydroxytamoxifen has a high binding affinity for the ER and is broken down into cis and trans forms. The cis isomer is has a predominantly agonistic function and an increased ratio of cis to trans isomer [227] has been detected in vivo in resistant tumours.

### **5.5.7 Agonistic Properties of Tamoxifen**

In breast cancer tissues tamoxifen acts mainly as an oestrogen antagonist however its agonist properties are widely reported in endometrium and bone. The agonistic properties of tamoxifen appear to be mediated by the oestrogen receptor beta isoform. This leads to increased transcription of genes which encode for growth promoting proteins. Human breast cancer cell lines exposed to long term tamoxifen become exquisitely sensitive to the growth promoting effects of oestrogen. In addition human breast cancers that become tamoxifen resistant can exhibit marked regression on tamoxifen withdrawal indicating a tamoxifen mediated pathway growth pathway. This effect could have a potential impact on the interpretation of results in sequential therapy trials.

### **5.5.8 Induction of Growth Factor Signalling Pathways and Their Ligands**

Studies have shown an increase in transforming growth factor alpha (TGF $\alpha$ ), a ligand for EGFR, in an oestrogen receptor positive cell line in the presence of oestradiol due to a direct transcriptional effect [230]. In another study immunohistochemical analysis of 51 breast cancers revealed increased levels of TGF $\alpha$  in 65% of tumours. TGF $\alpha$  levels were related to the endocrine sensitivity of the disease, with unresponsive tumours frequently showing high levels of TGF $\alpha$  immunoreactivity. This relationship was observed in ER positive disease and was independent of the EGFR status. This infers a role for the EGFR ligand, TGF $\alpha$ , in the development of endocrine insensitivity in ER positive breast cancer by mechanisms which appear independent of tumour growth fraction; the latter being determined by Ki67 immunostaining which is a marker of tumour cell proliferation [231]. Under basal conditions in an in vitro study of a TAMR MCF7 breast cancer cell line, phosphorylated EGFR/HER2, EGFR/HER3 but not HER2/HER3 receptor heterodimers were detected in association with increased levels of phosphorylated extracellular-signal regulated MAPkinase. Both cell lines (wild and TAMR) were capable of generating a range of EGFR-specific ligands and increased expression of TGF $\alpha$  was observed in TAMR cells. This highlights the role that TGF $\alpha$  plays in the generation of endocrine resistance through EGFR [232]. TGF $\alpha$  acts on the EGFR and also, in a paracrine fashion, on breast stromal cells to promote breast cancer cell growth via the insulin growth factor receptor (IGF1R). Several cell line studies have shown that tamoxifen stimulated cell proliferation is dependent on insulin-like growth factor I (IGF-1). This suggests that tamoxifen stimulates cell proliferation at the development of resistance by up

regulating the IGFR and sensitising the cell to the effects of oestrogen [233, 234]. The Tenovus group showed increases in epidermal growth factor receptor, MAP Kinase and AKT signalling at the development of tamoxifen resistance. There is some evidence that this is in part mediated by the insulin-like growth factor receptor pathway [235]. Thus both type I growth factors and the IGF1 pathway can be elevated at the development of, and may play a pivotal role, in the mechanism of tamoxifen resistance.

### **5.5.9 Recruitment of Downstream Effectors**

AKT, bcl2 and cfos are downstream effectors of the growth factor signalling pathways as discussed above. AKT and cfos levels are elevated at the development of tamoxifen resistance. TAMR cell lines express high levels of activated AKT-1 [176]. AKT inhibition with an n-3 fatty acid (EPA) has been shown to restore tamoxifen sensitivity [185]. Increased cfos expression is seen at the development of tamoxifen resistance and is also associated with the expression of growth factor receptors such as EGFR. Transcription for genes coding for cell metastasis and invasion is seen in association with elevated levels of cfos expression at the time of endocrine resistance [197, 198]. Although bcl2 expression is linked to ER and PgR expression and hence to an endocrine sensitive phenotype, diminished apoptotic response caused by bcl2 over expression is associated with cellular resistance to chemotherapeutic drugs [191] and has been implicated in the development of a metastatic phenotype [192, 193].

## 6 Development of Targeted Therapies “Gefitinib”

As there is now considerable evidence that the growth factor receptor pathways and their downstream signalling effectors are involved in the development of endocrine resistance, agents blocking these pathways should cause tumour regression. TAMR MCF7 cells treated with the EGFR tyrosine kinase inhibitor gefitinib show marked growth regression. In addition to this, preliminary studies combining these agents with conventional endocrine therapy seems to delay the development of acquired resistance and may even prevent de novo resistance [232, 236, 237].

### 6.1 *Biochemistry and Action*

ZD1839 (4-(3chloro—4-fluorophenylamine)-7-methoxy-6 (3-(4morpholinyl)quinazoline) “Gefitinib” is a potent non-cytotoxic anthraquinolone that selectively inhibits the epidermal growth factor receptor tyrosine kinase.(EGFR-TKI) [238]. It competitively inhibits ATP so causing inhibition of the tyrosine kinase enzyme and blocking the transcription of downstream growth promoting genes. This leads to the inhibition of ligand induced cell proliferation. The antitumour activity of ZD1839 in combination with other cytotoxic agents does not seem to require high levels of EGFR expression [239]. The basis for the development of this compound was the discovery of 4-aniloquinolones in 1994. Structure and activity relationship studies established that several specific substitutions within aniloquinazolines provided the most potent EGFR tyrosine kinase inhibitors in vitro [240]. These substitutions included electron donating substituents at the 6- and 7- positions of the quinazoline; a lipophilic substituent at the meta-position of the aniline; a free

NH at the 4- position and a free CH at the 2-, 5-, and 8- positions. Several derivations were investigated; compound 4 had a substitution with chlorine in place of the methyl group and fluorine at the para-position of the aniline. This substance had improved efficacy on oral dosing in mice and reduced clearance. It was potent and metabolically stable and became the focus for further development. A series of modifications to the alkyl groups of the methoxy side chains lead to improved in vivo activity.

## **6.2 Trials**

Gefitinib (ZD1839), a direct derivative of compound 4, was chosen for drug development because it achieved high and sustained blood plasma levels over a 24-hour period and was most compatible with once daily dosing.

Pharmacokinetic studies revealed that ZD1839 was fairly slowly absorbed taking 7 – 10 days to reach steady state levels with daily dosing [241]. It inhibited the growth of human cancer xenografts in a dose dependant manner with marked regressions seen in some tumours [242].

The IC<sub>50</sub>, or the half maximal inhibitory concentration, represents the concentration of an inhibitor that is required for 50% inhibition of its target. In simpler terms, it measures how much of a particular substance/molecule is needed to inhibit some biological process by 50% in vitro. IC<sub>50</sub> is commonly used as a measure of drug potency of antagonist drugs in pharmacological research. The IC<sub>50</sub> for EGFR was 0.033 micro moles [243] and the IC<sub>50</sub> for HER2 was > 3.7 micro Moles [244].

Investigations into the sensitivity of breast cancer cells to ZD1839 have demonstrated that whilst parental hormone sensitive cells are relatively insensitive to this molecule, the growth of endocrine resistant variants is

inhibited by ZD1839 via an inhibition of EGFR autophosphorylation and the MAP kinase signalling pathway. These responses to the EGFR inhibitor are long lasting (greater than 3 months), implying that the switch to using EGFR signalling is an important survival mechanism for the resistant cells [133]. Administration of the drug during the period in which endocrine resistance normally develops can result in additional cell loss and a very significant delay in the development of the endocrine resistant phenotype. Studies have shown growth arrest for longer than 6 months mediated via reduced cellular proliferation and increased cell death [232].

### **6.3 *Preclinical Studies***

Preclinical studies and on-going clinical trials have demonstrated mechanism-based, predictable, and reversible anti-tumour activity and toxicity.

#### **6.3.1 Animal pharmacokinetics**

The major route of excretion for ZD1839 and its metabolites is via the bile. ZD1839 is extensively metabolised to a number of components, extensively distributed outside the central compartment and rapidly cleared. Bioavailability following oral dosing is approximately 50% [245].

Exposure to ZD1839 increases approximately proportionally with dose. The plasma concentration-time profile data show evidence of prolonged absorption occurring at the highest doses.

#### **6.3.2 Animal toxicology**

ZD1839 showed no genotoxic potential in-vitro. The NOAEL (no observable adverse effect level) is the highest level of a substance to which test animals

are exposed on an ongoing basis that causes no significant adverse toxicological effects. After administration of ZD1839 for up to 1 month is 10 mg/kg per day; at 6 months it is 1 mg/kg per day.

The most common form of toxicity was epithelial and included inflammation of eyelids, folliculitis, and degeneration of hair follicles. The findings at the lowest tested dose level were similar to those in the top and intermediate dose levels when given for longer but were less severe and had a lower incidence.

Reversible ocular changes included granular/rough appearance to the cornea and corneal translucency without ulceration. Irreversible corneal opacities were seen only in the dog at the highest dose given chronically for 6 months. Rarely, renal papillary necrosis was seen at the highest dose level, but again only with prolonged exposure. Rare papillary microlithiasis at higher doses was seen only in rats [246].

In addition, electrocardiogram (ECG) recordings revealed a rare lengthening of the PR interval, with large variations between the individual PR interval measurements. A second-degree atrio-ventricular block occurred in one instance. ECG findings returned to normal when therapy was discontinued.

The ophthalmologic, renal, and skin changes were considered to be related to the pharmacological activity of ZD1839. Cardiac change was considered a possible effect of ZD1839 [247].

Biochemical or haematological abnormalities included increased white blood cells, decreased red cells, reduced plasma albumin, and increased plasma liver enzymes (alkaline phosphatase [ALP], alanine transaminase [ALT], and aspartate transaminase [AST]). They were generally reversible on

discontinuation of the drug. The ovaries showed a reduction in the number of corpora lutea.

## **6.4 ZD1839 Clinical Experience**

### **6.4.1 Clinical Pharmacokinetics**

ZD1839 absorption in man is moderately slow with plasma concentrations typically reaching a maximum between 3 and 7 hours after dosing. Beyond the peak the concentrations decline in a biphasic manner, with a terminal half-life between 27 and 51 hours in healthy volunteers, and 27 to 85 hours in cancer patients. Data from healthy volunteers show that the area under curve in the first 24 hours after dosing [AUC (0-24)] represents approximately 50% of the total AUC and that urinary recovery of ZD1839 is low, indicating that renal excretion is not a major route of elimination for ZD1839 in man. Administration of oral doses of ZD1839 to healthy volunteers in the fed state results in a reduction in exposure to the drug that is not considered to be clinically significant. On limited multiple dosing to volunteers, the pharmacokinetics of ZD1839 have been shown to be predictable, and it is anticipated that, based on 24 hourly dosing, steady state would be achieved within 7 to 10 days [248-250].

### **6.4.2 Clinical Trials with ZD1839**

Phase I clinical trials are designed to determine the appropriate dose of ZD1839 in the population to be treated. They include patients with advanced disease considered refractory to standard treatment regimens. The dose is increased as more patients are entered onto the trial to determine the maximum tolerated dose. Dose limiting toxicity is described as any drug related toxicity



greater than or equal to grade 3 or 4 by common toxicity criteria (CTC), significant corneal epithelial change or PR interval prolongation in 2 or more patients at any particular dose level.

To date, many cancer patients have received oral ZD1839 in Phase I, and II trials. The doses tested range from 50 mg to 1,000 mg. The majority of these received a dose of 250mg per day. The 2 largest Phase I trials in which ZD1839 was given daily without interruption, had a combined total of 142 enrolled patients, and completed enrolment at the highest planned dose level of 1,000 mg. At this 1,000 mg dose level, CTC grade 3 diarrhoeal dose limiting toxicity was reported in 4 patients. Increasing intolerability with dose interruptions was seen at doses of or greater than 600 mg daily.

In these Phase I trials, consistently observed dose-related, mechanism-based toxicity was common and confined to the skin and gastrointestinal system; rare hepatic enzyme elevation has also occurred. There was a notable lack of marrow toxicity that reflects the absence of EGFR on mature haematopoietic cells. Skin toxicity consisted mainly of a CTC grade 1-2 pustular rash on an erythematous base; gastrointestinal toxicity consisted mainly of CTC grade 1-2 loose or watery, intermittent non-bloody, non-mucoid stools occasionally with nausea or isolated episodes of vomiting and was less commonly seen. The majority of patients with rash at higher doses also experienced diarrhoea. Skin, gastrointestinal and the rare hepatic toxicity rapidly reverse with drug discontinuation and/or symptomatic support. Consistent or drug related haematopoietic, renal and corneal toxicity have not been reported. Uveitis occurred in one patient. In 2 studies, 8.2 % of patients experienced mild, transient adverse events related to the eye that were considered to be possibly

related to trial therapy (e.g. transient redness or itchiness). Four cases of reversible corneal erosion have been reported (accompanied by hyperaemia in 2 of the cases). Three of these cases were directly related to aberrant eyelash growth and one to a possible ocular foreign body. In 3 of the 4 patients the condition reversed within 1 week. These adverse events happened with long-term dosing (3 to 7 months) at higher doses (400, 600 and 800 mg).

All but 1 of the patient deaths were considered by investigators as due to disease progression; 1 patient's death was considered by investigators as possibly drug related and at autopsy a large, fatal pulmonary embolus was found [251].

These studies demonstrated that ZD1839 was well tolerated. The dose limiting toxicity seen was diarrhoea at the 700mg per day dose. Encouraging antitumour activity was seen across a wide range of tumour types especially in Non Small Cell Lung Cancer (NSCLC).

In another Phase I clinical trial no dose limiting toxicity was seen in a maximum dose of 800mg per day [252]. The range of side effects and responses was similar. Similar toxicity profiles and anti-tumour activity have been reported in initial clinical trials with other EGFR tyrosine kinase inhibitors.

Interstitial lung disease (ILD) or drug induced lung injury is being increasingly recognised and is associated with a wide range of agents including methotrexate, cyclophosphamide and other chemotherapeutic agents. ILD, including interstitial pneumonitis, is a common complication of lung diseases including advanced lung cancer, regardless of treatment. It has been widely observed in clinical trials in which chemotherapy and/or radiotherapy has been

used for the treatment of advanced lung cancer. Hypersensitivity pneumonitis is probably the most commonly described pattern of 'classic' drug-associated ILD [253]. It has been uncommonly described yet widely reported in patients treated with ZD1839, with a worldwide frequency of less than 1%. In Japan the frequency was 2%, in the rest of the world only 0.3%. This is lower than the frequency reported for other lung cancer therapies. ILD had a fatal outcome, whether deemed ZD1839-related or not, in approximately 0.24% in this group of over 50,000 patients receiving ZD1839. The occurrence of pulmonary toxicity and interstitial lung disease was similar across all treatment arms in the placebo-controlled INTACT trials.

The clinical picture is of a fairly acute onset of dyspnoea sometimes associated with cough or low-grade fever. This could become quite severe within a short period of time and usually resulted in hospitalisation. Radiological investigations, often including high resolution CT scan, frequently showed pulmonary infiltrates or interstitial shadowing with ground glass appearance. There was often respiratory distress with arterial oxygen desaturation. Cultures were frequently negative for bacterial growth. In a number of cases, the event did respond to corticosteroid therapy but this was not always so and a significant number of cases have had a fatal outcome [249]. The commonly seen histopathological patterns of drug-associated lung injury include pulmonary oedema, diffuse alveolar damage, nonspecific interstitial pneumonia, bronchiolitis obliterans organising pneumonia, eosinophilic pneumonia and pulmonary haemorrhage. The pattern most commonly seen with gefitinib use is diffuse alveolar damage and pre-existing idiopathic pulmonary fibrosis appears to have a detrimental effect on outcome.

(AstraZeneca and Iressa Expert Committee 2003). It is recommended that patients receiving gefitinib are regularly monitored and that therapy should be discontinued immediately should this occur.

In summary Phase I trials with ZD1839 have demonstrated that tolerability of oral ZD1839 is dose dependent, with predictable, mild, reversible, mechanism-based gastrointestinal and skin toxicity at doses below 600 mg. Moreover, significant clinical activity with tumour regression or disease stabilization was seen in a variety of cancers [254]. Unlike safety, activity signs were seen across a wide range of doses, from 150 mg to 800 mg.

Phase II clinical trials are designed to attempt to measure the biological response of a particular tumour to a specific treatment. Typically patients with a tumour for which there is no known effective treatment are included. Combination regimens may also be evaluated in Phase II clinical trials. The goal is to ensure that treatment is safe, feasible and promising enough to move onto the next phase of expansion.

A Phase II clinical trial with gefitinib in NSCLC ( IDEAL 1 and IDEAL 2 ) recruited patients who had previous chemotherapy regimes and randomised them into 2 groups with dosages of either 250mg per day or 500 mg per day ( n = 426 ). Comparable efficacy was seen within the two groups, but the side effect profile was lower with the lower dose [255].

Phase III clinical trials are designed to compare an experimental treatment to an accepted standard of care and evaluate endpoints such as survival and symptom control. These trials are typically performed in multicentre settings. INTACT 1 & 2 investigated ZD1839 at 250 or 500 mg per day in combination with conventional chemotherapy regimes in advanced NSCLC. A total of 2130

patients were recruited to 3 arms. The regimes were gemcitabine + cisplatin or paclitaxel + carboplatin. Within these arms patients were randomised to ZD1839 at 250 mg per day, 500mg per day or placebo. These studies showed that ZD1839 showed no survival benefit when combined with standard platinum based chemotherapy [256].

### **6.4.3 ZD1839 and Breast Cancer**

There have been several Intention to Treat trials with gefitinib in breast cancer encompassing DCIS prevention, pre-surgical, neo-adjuvant and metastatic disease with conflicting results. ZD1839 has been studied in combination with other drugs and as a monotherapy in 441 patients. In the initial trials with gefitinib as monotherapy in post-menopausal metastatic oestrogen receptor positive breast cancer the majority of patients were heavily pre-treated with chemotherapy, were not selected for EGFR positivity and the response rates were poor. Baselga et al studied 32 patients and had a median time to progression (TTP) of 8 weeks with a 38% CB rate [257] while Albain et al treated 63 patients and had a clinical benefit (CB) rate of 5% [258]. The Australian Clinical Trials Group [259] enrolled 66 women with advanced breast cancer: 39 whose breast cancers had stopped responding to hormone therapy and 27 whose tumours were ER-negative and PgR-negative. They found no gefitinib responses after 28 weeks of treatment and the study was abandoned. Further EGFR TKI studies are ongoing, but to date, a phase II trial of erlotinib (as monotherapy of 150 mg/day) in heavily pre treated locally advanced or metastatic breast cancer again exhibited only modest responses [260]. Further investigation into combining erlotinib with convention chemotherapy for NSCLC has again failed to show a benefit [261]. The

TRIBUTE trial is a multicenter, randomized, double-blind phase III trial of erlotinib 150mg orally once daily plus standard carboplatin and paclitaxel vs. chemotherapy alone for the first-line treatment of advanced NSCLC.

TRIBUTE is expected to enroll 1050 patients, and the primary end point is survival.

## 7 Aims of Thesis

In cell culture studies both TAMR MCF7 cells and oestrogen receptor negative cell lines express high levels of EGFR and are growth inhibited by gefitinib. Our hypothesis is that this cell culture work may be translated into the clinical setting providing a further therapeutic agent in the treatment of breast cancer. It is anticipated that gefitinib may show activity in both oestrogen receptor positive and oestrogen receptor negative breast cancer forming a valuable addition to our armamentarium in these patients.

This thesis aims to compare the efficacy and tolerability of a new tyrosine kinase inhibitor – gefitinib (ZD1839) in two separate and distinct patient groups. Group 1 – designated acquired tamoxifen resistant, will be oestrogen receptor positive and will have either developed recurrent disease whilst on, or < one year after cessation of, adjuvant tamoxifen therapy, or progressed in their existing recurrent disease whilst on tamoxifen therapy. Group 2 – designated de novo resistant will be oestrogen receptor negative and will have received no more than one prior chemotherapy for advanced disease. Neither group will have received prior aromatase inhibitors, either as adjuvant therapy or as treatment for recurrent disease.

In the clinical setting tamoxifen will be ceased in the face of treatment failure due to concerns regarding its potential agonist effects. It is possible that any gefitinib effect seen here may be due to tamoxifen withdrawal. To explore this issue and in contrast to the clinical study, a murine model will be used to create tamoxifen resistant xenografts. The tumours will be treated with tamoxifen alone (designated control) or tamoxifen + gefitinib (designated treatment) in an effort to investigate the true gefitinib effect on tumour growth.

Tumour tissue will be obtained from the patients in the clinic at designated time points and analysed for predictors of response to treatment and/or failure. Biological parameters will be measured whilst on treatment to investigate oestrogen receptor and type 1 growth factor receptor pathways and their downstream signalling factors (ER, PgR, EGFR, pEGFR, HER2, pHER2, IGFR, pIGFR, pMAPKinase, pAKT, Ki67, pSer118, pSer167, cfos and bcl2.) We will examine the change seen from one pathway to another during acquired tyrosine kinase inhibitor resistance and potential crosstalk between these pathways.



## **8 Materials and Methods**

### ***8.1 In Vivo Mouse Xenograft Work***

#### **8.1.1 Background**

The MCF7 breast adenocarcinoma cell line was cloned from the pleural effusion of an advanced breast cancer patient in 1970 [262]. It was found to express high levels of the oestrogen receptor and has been used extensively in the study of tamoxifen response and the development of resistance [263]. Parental or “wild type” MCF7 cells are oestrogen receptor positive and respond to hormonal manipulation. However on prolonged oestrogen deprivation (6 months duration), due to continuous exposure to 4-hydroxytamoxifen at a concentration of 100nM, the cell line begins to proliferate again leading to a tamoxifen resistant (TAMR) cell line. This resistant cell line expresses up to 10 fold higher levels of EGFR and also expresses numerous EGFR ligands which are able to activate EGFR and HER2 thus promoting oestrogen independent cell growth [264]. Preclinical studies have shown that the growth of TAMR MCF7 cells in culture can be slowed by the administration of gefitinib at 1 micro molar concentration [264, 265] and that the duration of response can be prolonged [159]. The study was initiated to develop a TAMR tumour xenograft from MCF7 cells gifted from the Tenovus institute in Cardiff and to expose it to tamoxifen (designated control) or tamoxifen + gefitinib (designated treatment).

Nude (nu/nu) athymic immuno-compromised MF1 mice have been used extensively to generate human tumour xenografts and remain the experimental method of choice for testing anti-tumour efficacy of new compounds prior to administration in man. In general xenografts are relatively easy to generate and

are inexpensive. However human breast cancer is one of the more difficult xenografts to create with a reported success rate of 7 – 20%. The take rate is affected by the age and strain of mice used, the site of transplantation and whether or not oestrogen supplementation is used [266]. Solid tumours are formed reasonably quickly after the subcutaneous placement of MCF7 breast cancer cells into the flank [267]. The tumour growth rate is accelerated compared to the clinical setting to achieve xenografts that grow rapidly to compensate for the mouse limited life span.

The route of administration of the test substance (gefitinib) and control (tamoxifen) in this study was by the oral route or a subcutaneously implanted pellet as outlined in the individual protocols. The xenografts were given 10 days to establish themselves before treatment began. Tamoxifen can be given as an oral preparation, an intraperitoneal injection or as a subcutaneous pellet [268-270]. Gefitinib was given as an oral preparation, the vehicle was 0.1% Tween-80 made up to 0.2mls. Female nude mice were used for all studies and these were bred within the Academic Unit of Cancer. The study was located in the Academic Unit of Cancer Studies (AUCS) Level 2 containment facility, F Floor, Medical School, University of Nottingham.

### **8.1.2 Husbandry**

The mice were maintained in sterile isolators within a barriered unit illuminated by fluorescent lights set to give a 12 h light-dark cycle (on 07.00, off 19.00), as recommended in the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. The room was air-conditioned by a system designed to maintain an air temperature range of  $26 \pm 2^{\circ}\text{C}$ . The mice were housed in groups of 5 or 8 during the procedure in plastic cages (Techniplast

UK) with irradiated bedding and provided with both nesting materials and environmental enrichment. Sterile irradiated R/M 3 diet (Dietex International UK, product code 831200) and autoclaved water were offered *ad libitum*.

### **8.1.3 Animal Welfare and Identification**

An experienced technician checked the condition of the mice daily. Unexpected adverse effects were noted and detailed in the final report and also reported to the Named Animal Care and Welfare Officer (NACWO) and Named Veterinary Surgeon (NVS). Animals could be terminated at any time during the study if the tumour size became excessive or any unexpected adverse effects were noted according to Home Office Project Licence PPL 40/2323.

Each animal was allocated a unique identification number by implantation of a transponder (Microchip Identification Devices – Fingerprint UK). This number appeared on the data sheets.

### **8.1.4 Experimental Procedure**

#### **8.1.4.1 General cell maintenance protocol**

All of the following steps were conducted two to three times weekly in a sterile environment using aseptic techniques.

The tissue culture flask (25 or 75cm<sup>2</sup> area) was examined under a low power microscope to assess the degree of confluence of the cell monolayer (confluence refers to the extent of coverage of the cells over the available surface area). Generally, depending on the cellular growth rate, flasks exhibiting a greater than 70% confluence should be split to reduce cell numbers. Flasks with a confluence lower than 70% were re-fed with growth

media (1640 RPMI, supplemented with 10% heat-inactivated Foetal bovine serum (FBS) and 2mM l-glutamine).

To re-feed, the used media was aspirated from the flask and re-fed with 15-20ml of growth media (pre-warmed to room temperature). The flasks were replaced in the incubator (37°C, 5% CO<sub>2</sub>) and the tops slightly loosened to allow the CO<sub>2</sub> to permeate the flask environment

To split the cells, the used media was aspirated from the flask and 2ml of 0.025% EDTA/PBS (phosphate buffered saline) was added. The flasks were incubated at 37°C for 5-10 minutes to allow the cells to detach. A Pasteur pipette was used to remove any remaining attached cells by flushing the area with the EDTA. Finally approximately  $\frac{3}{4}$  of the cell suspension was removed and the flask re-fed with 15-20ml growth media (any left over cells were frozen down, used to expand the cell line or discarded). The flasks were replaced in the incubator (37°C, 5% CO<sub>2</sub>) and the tops slightly loosened again to allow the CO<sub>2</sub> to permeate the flask environment.

The tamoxifen sensitive (wild type) cell line was maintained in red medium which is itself oestrogenic. The tamoxifen resistant media was RPMI supplemented with 5 or 10% FBS

#### **8.1.4.2 Preparation of cells for *In vivo* use**

For transfer to *In vivo*, cells must be no more than 80% confluent. The flasks were carefully examined to ensure that there were enough flasks of sub-confluent cells to harvest enough cells

To split the cells, the used media was aspirated from the flask and 2ml of 0.025% EDTA/PBS (phosphate buffered saline) was added. The flasks were incubated at 37°C for 5-10 minutes to allow the cells to detach. A Pasteur pipette was used to remove any remaining attached cells by flushing the area with the EDTA. The harvested cells were pooled into a labelled sterile universal and the cell suspension topped up 25ml with growth media.

The universal was centrifuged for 5 minutes at 1500RPM. The media was aspirated off and the cell pellet re-suspended into 10ml assay media. The cell suspension was gently passed through a green gauge needle 2 – 3 times to ensure a single cell suspension. An equal volume mixture of cell suspension and trypan blue (a 4:1 mixture of 0.2% trypan blue and 4.25% saline) was prepared and counted using a Neubauer haemocytometer (non-viable cells stain blue allowing viability to be assessed). Cells must be 90% viable or above for *In vivo* transfer. The universal was again centrifuged for 5 minutes at 1500RPM. The media was aspirated off and the cell pellet re-suspended into sterile PBS (pH7.2 at a concentration of  $1 \times 10^7$ /ml). The cell line was injected into the subcutaneous tissue of the flank of the donor mice.

Female animals were anaesthetised using an appropriate anaesthetic. The cell suspension, in a volume of 50-100 microlitres was injected subcutaneously into the flank. As the tumour line was oestrogen dependant, oestrogen pellets were

implanted (1.5mg oestradiol). The tumours were expected to grow within 21-30 days.

The tumour cells were maintained in serial passage in nude mice. For the therapy studies, the donor mice were sacrificed and tumours excised. The tumour was finely minced, and 3mm<sup>3</sup> sections were implanted subcutaneously in to the flank of the mice under anaesthesia (Hypnorm, Roche/Nypnovel Janssen). Animals were examined regularly for the appearance of tumours. When measurable tumours had been established in the majority of mice, tumour size was measured three times weekly using callipers. Each mouse continued on study until the tumour size (as specified in the Home Office licence) or other clinical signs, necessitated removal of that mouse from the study. Tumour dimensions were recorded (length and width) and tumour cross-sectional areas calculated. Animals were terminated at any time during the study if the tumour size became excessive or any adverse effects were noted according to Home Office Project Licence PPL 40/2323.

At the end of the study bromodeoxyuridine (5mg/mouse) was administered. Animals were terminally anaesthetized with Hypnorm (Roche)/Nypnovel (Janssen). Tumours were removed, weighed and cut in half and representative samples were placed into histology cassettes and fixed in formal saline.

Any animal found dead or killed prematurely during the study was subjected to a necropsy, at the discretion of the Study Director. A macroscopic examination was performed, after opening the thoracic and abdominal cavities, by observing the appearance of the tissues *in situ*. Any abnormalities were recorded.

### **8.1.4.3 Toxicology Studies**

Toxicology studies were carried out prior to the commencement of the main studies. After discussion with Dr Wakeling at AstraZeneca mice were orally dosed with tamoxifen 3mg/kg per day and ZD1839 at 100mg/kg per day both contained in oral vehicle. The mice quickly began to develop side effects from the dosing at this level. They developed an acneiform rash concentrated mainly on the face and upper torso. They became lethargic and lost weight despite having their diet supplemented with mash. These side effects had been seen in a previous study within the unit and were felt to be attributable to the ZD1839. At this point, after discussion with AstraZeneca, the daily oral dosage of ZD1839 was decreased to 50mg/kg per day. Acceptable toxicity was experienced at this dose.

### **8.1.4.4 Data Analysis**

The tumour dimensions measured over the period of the study - length (L, long) and width (W, short) in mm were recorded and kept as the raw data on the CD. Plots of mean tumour growth curves were performed. Body weight data was also reported. The statistical analysis package SPSS 17 was used for all analysis and graph production.

A random coefficient model analysis was performed to allow for variance within the mouse treatment groups and also to calculate the treatment effect. Ki67 staining was assessed in 5 mice from each treatment group sacrificed after 14 days on therapy. Differences in mean Ki67 staining were assessed using a Mann Whitney U test. A value of  $p \leq 0.05$  was considered significant

## **8.2 Experimental Design**

### **8.2.1 Establishing Xenografts**

Wild type (tamoxifen sensitive) and TAMR cells arrived from Tenovus in April 2002. Both TAMR and Wild type MCF7 were extremely slow to grow in culture despite no change in culture medium. Sufficient cells were obtained to inject into donor mice in May 2002. Wild type MCF7 from Tenovus did not produce tumour nodules in these donors therefore an in house wild type MCF7 cell line was used. Both cell lines were injected into donor mice but no growth of xenografts occurred. Throughout the months of May, June and July a further 3 attempts to create xenografts were made. Eventually at the end of August a TAMR tumour nodule began to form and by the beginning of September there were TAMR xenografts in 7 out of 12 mice. These mice were sacrificed and the tumour fixed in paraffin blocks. The tumours were analysed for EGFR expression to be certain that there had not been a phenotypic change in the cells during the process. Tumour nodules were finally created from the in house wild type MCF7 cells in October and xenografts established in January 2003.

### **8.2.2 Mouse Work Time Scales**

#### **8.2.2.1 Study 1**

The first study used the xenografts derived from the wild type tamoxifen sensitive cell line from The University of Nottingham. Tamoxifen was initially administered orally at a dose of 3mg/kg per day. ZD1839 was administered at a dose of 50mg/kg per day. There were 10 mice each in groups 5, 6 and 7. There were 20 mice in group 8 (Table 1). No effect was seen on xenograft growth from tamoxifen alone. This was thought most likely to be due to suboptimal



tamoxifen dosing. The tamoxifen dose was increased to 10mg/kg/day at day 41 in 5 of the mice in group 5 (now designated 5A) .The study ran for 53 days.

Group 5	Untreated Controls
Group 5A	Treated from day 41 with tamoxifen 10mg/kg
Group 6	Gefitinib 50mg/kg
Group 7	Gefitinib 50mg/kg + tamoxifen 3mg/kg
Group 8	Tamoxifen 3mg/kg

Table 1: Xenograft Wild Type Tamoxifen Sensitive Study 1 Design

### 8.2.2.2 Study 2

The second study used the xenografts created from the gifted TAMR MCF7 cells. As a result of the problems experienced with the tamoxifen dosing in the previous study these were treated with tamoxifen 5mg per 60 day release subcutaneously or tamoxifen + gefitinib 50mg/kg/day per day orally (Table 2). There were 15 mice in each group. A third of these were sacrificed at two weeks on treatment and the tumours excised, paraffin fixed and analysed for Ki67 expression. The study ran for 73 days

Group 1	Tamoxifen 5mg per 60 day release
Group 2	Tamoxifen 5mg per 60 day release + gefitinib 50mg/kg/day orally

Table 2: Xenograft Tamoxifen Resistant Study 2 Design

## **8.3 Clinical Work**

### **8.3.1 Background**

A Phase II clinical trial was required to further investigate the efficacy and safety of ZD1839 in patients with breast cancer who have acquired resistance to tamoxifen or have ER negative tumours. In addition, an exploratory study was conducted to investigate the relationship between EGFR expression and anti-tumour response; because ZD1839 inhibits EGFR tyrosine kinase directly, rather than through an extra cellular indirect approach such as an EGFR monoclonal antibody, it is not known if over-expression, and not simply expression, is needed for treatment efficacy. Previous studies have suggested that the level of EGFR expression has no bearing on the response to treatment [239]. Biological marker studies were conducted to assess the biological changes induced in breast cancer by ZD1839.

### **8.3.2 Trial Design**

This was a phase II trial of two distinct groups of patients with breast cancer. Group 1 – designated acquired tamoxifen resistant, were oestrogen receptor positive and had either developed recurrent disease whilst on, or < one year after cessation of adjuvant tamoxifen therapy (n=14), or progressed in their existing recurrent disease whilst on tamoxifen therapy (n=14). Group 2 – designated de novo resistant, were oestrogen receptor negative and had received no more than one prior chemotherapy for advanced disease (n=26). Neither group had received prior aromatase inhibitors, either as part of their adjuvant therapy or as treatment for recurrent disease. This was to ensure that the two groups were relatively treatment naïve. They were treated daily with

ZD1839 (500 mg/day) initially for a period of 6 months. A total of 54 patients were recruited from the City Hospital, Nottingham over a period of months from the Locally Advanced Primary Cancer Clinic, the Advanced Breast Cancer Clinic and the Elderly Primary Cancer clinic. Initial recruitment planned 27 oestrogen positive acquired tamoxifen resistant patients and 27 oestrogen receptor negative de novo resistant patients. In fact one of the patients designated oestrogen receptor negative had had a long period of CB on tamoxifen therapy and, although her pre-tamoxifen biopsy had been oestrogen receptor negative, her subsequent biopsies on tamoxifen were oestrogen receptor positive. Hence she was reclassified into the oestrogen receptor positive tamoxifen resistant group. Giving a final 28 oestrogen receptor positive acquired tamoxifen resistant patients and 26 oestrogen receptor negative de novo resistant patients.

#### Primary endpoints

1. Objective tumour response (complete + partial response) based on modified Union Internationale Centre le Cancer/World Health Organisation (UICC/WHO) criteria [96]. Tumour assessment was done every 4 weeks after start of treatment, then every 12 weeks from 6 months onwards. Lesions were assessed using the same methods on each occasion. Initial tumour assessment was performed within 14 days before starting ZD1839. In patients with breast disease assessment was by clinical examination using callipers to determine bi-dimensional product and/or ultrasound examination as deemed appropriate. In patients with measurable lung disease, chest X-ray or computed tomography (CT) scans were required. In patients with non-measurable

but evaluable pulmonary disease chest x-rays were required. Liver ultrasound or CT scans were required for patients with newly detected or previously diagnosed liver metastases at the specified tumour assessment times. If bone metastases were present radiographs of involved bones were obtained and repeat radiographs obtained at protocol specified tumour assessment times.

2. Clinical benefit. Disease control rate was based on objective tumour assessments and included those patients with a best overall response of CR or PR, plus those with SD that was sustained for at least 24 weeks from initiation of therapy. Patients with evidence of progressive disease at 4 and/or 8 weeks could continue on study medication up to a maximum of 12 weeks on condition that all the following criteria were met:
  - The progressive disease was not immediately life threatening requiring chemotherapy as assessed by the study site multi-disciplinary team.
  - That it was felt that it would be reasonable to continue the patient on ZD1839 for a further, defined, period.
  - Patients would be informed that their disease had progressed and would only be continued on ZD1839 with their full consent. Patients would be asked to sign a short statement in their case notes indicating that they consent to continue on ZD1839 until their 8 or 12 week assessments.
  - The clinician would be free to withdraw the patient from the study at any time and change therapy.
  - Evidence of progressive disease at 12 weeks would be a definite indication to discontinue ZD1839 and change treatment.

3. Frequency and severity of adverse events (AEs). An adverse event was defined as “the development of an undesirable medical condition or the deterioration of a pre-existing medical condition following or during exposure to a pharmaceutical product, whether or not considered causally related to the product”. All adverse events including causality assessment were collected. Patients were monitored for AEs during the trial and for 30 days after the last dose of trial drug. Any serious AEs within 30 days after stopping the trial drug were followed to resolution unless the condition was unlikely to resolve because of the patient’s underlying disease. Any CTC grade 3 or 4 haematology or biochemistry laboratory value considered not due to tumour progression were recorded as an AE. AEs and laboratory values were graded according to the well established NCI CTC (Version 2.0) and recorded.

### **8.3.3 Secondary endpoints**

1. Progression free survival. Progression free survival or time to progression (TTP) was assessed from the date of entry to the study to the date when objective disease progression was observed. Death was regarded as a progression event in those patients who died before disease progression. Patients without documented objective progression at the time of analysis were censored at the date of their last objective tumour assessment.
2. Duration of response. Duration of response (DoR) was assessed for each patient with a best objective tumour response of CR or PR. DoR was defined as the interval between the date of first documented

response and the date of objective, documented disease progression for these patients

#### **8.3.4 Exploratory endpoint**

1. EGFR expression. Tumour tissue samples were analysed to measure the level of EGFR expression using a well established scoring system – the H Score. Patients who were simultaneously evaluable for tumour response and EGFR expression were included in the analysis to assess if there was a correlation between EGFR expression and tumour response.
2. Effects of ZD1839 on biological markers. These were assessed on sequential tumour biopsies before and during treatment and at progression

### **8.3.5 Inclusion Criteria**

The patients met all of the following to be considered for the trial

1. Histological or cytological confirmation of breast cancer that is either a primary tumour in a patient unfit for or who has declined surgery or is advanced (locally or metastatic) disease
2. acquired resistance to tamoxifen or an oestrogen receptor negative tumour
3. no previous aromatase inhibitor
4. at least one measurable or assessable lesion
5. WHO performance status 0 - 2
6. life expectancy of 12 weeks or more
7. age 18 years or older
8. written informed consent to participate in the trial.

### **8.3.6 Exclusion criteria**

Any one of the following excluded a patient from entering the trial:

1. more than one previous chemotherapy regimens for advanced disease
2. prior anthracycline chemotherapy ( $> 250 \text{ mg/m}^2$  adriamycin)
3. radiotherapy completed within 14 days prior to Day 1 of treatment
4. incomplete healing from prior oncologic or other major surgery
5. signs of neurological symptoms consistent with spinal cord compression

6. any evidence of clinically active interstitial lung disease (patients with chronic stable radiographic changes who are asymptomatic need not be excluded).
7. in the opinion of the investigator, any evidence of severe or uncontrolled systemic disease, (e.g., currently unstable or uncompensated respiratory, cardiac, hepatic, or renal disease) or evidence of any other significant clinical disorder or laboratory finding which makes it undesirable for the patient to participate in the trial
8. neutrophils less than  $1.5 \times 10^9$ /liter (L) or platelets less than  $75 \times 10^9$ /L
9. serum bilirubin greater than 2 times the upper limit of reference range (ULRR)
10. alanine amino transferase (ALT) or aspartate amino transferase (AST) greater than 2.5 times the ULRR if no demonstrable liver metastases or greater than 5 times the ULRR in the presence of liver metastases
11. serum creatinine greater than 2 times the ULRR
12. risk (in the investigator's opinion) of transmitting human immunodeficiency virus (HIV) or hepatitis B through blood or other body fluids
13. pregnancy or breast feeding (women of child-bearing potential)
14. patient was taking another systemic anti-cancer treatment
15. in the opinion of the investigator, any evidence of superior vena cava syndrome



16. known severe hypersensitivity to ZD1839 or any of the ingredients of this product
17. concomitant use of phenytoin, carbamazepine, rifampicin, barbiturates, or St John's Wort
18. treatment with a non-approved or investigational drug within 30 days before Day 1 of study treatment

### **8.3.7 Dosing Schedule**

Initially patients received a loading dose on day one of 1000mg (in two divided doses), this was subsequently abandoned due to a higher incidence of grade 2 CTC facial rash and grade 2 CTC diarrhoea.

Patients were treated daily with 500 mg ZD1839 at the beginning of the trial however adverse events, dose interruptions and dose reductions were common. In patients who developed significant toxicity consideration was first given to dose interruption. If any of the following conditions occurred, administration of ZD1839 was interrupted for a maximum of 14 days to allow the AE to resolve or decrease in severity:

1. CTC grade 3 or greater or unacceptable toxicity e.g. cosmetic effect of grade 2 rash
2. There was no consideration and/or corroborative evidence that the AE is due to progressive disease
3. The AE was consistent with previously described ZD1839 toxicity

At a minimum, re-assessment of toxicity was done twice weekly and more frequently if clinically indicated. Once the AE decreased in severity to CTC

grade 1 the patient could recommence at the 500mg dose. If the AE resolved to grade 2 the investigator may elect to decrease the patient's dose.

If a patient was re-challenged at the same dose and the same or other toxicity, at the same CTC grade or greater recurred, a second interruption of administration of the trial drug was allowed but the daily dose was then reduced. The dose reduction was by 50%, the dose decreasing from 500 mg to 250 mg. Only 1 dose reduction due to unacceptable toxicity per patient was allowed. The dose was continued until disease progression or withdrawal criteria were met. Patients who achieved clinical benefit (i.e. CR, PR or SD) on ZD1839 continued treatment until disease progression or withdrawal criteria were met.

Before entering the trial, patients were assessed to ensure that the eligibility criteria were met. Every patient provided written informed consent to the trial procedures (Appendix 1). When the data regarding interstitial lung disease became available it was incorporated into the trial consent form and all existing patients were re-consented to remain on trial.

The following were assessed within 14 days prior to the date of commencement of trial medication

1. Patient demography
2. Past medical history (i.e., all significant conditions that existed previously and were now resolved)
3. Details of previous cancer treatment
4. ECG

5. Patients who had received limited anthracycline treatment (i.e. less than 250mg/m<sup>2</sup>) or radiotherapy to the (left) breast/chest wall, had a trans-thoracic ECHO and LVEF in addition to an ECG
6. Full tumour assessment
7. Palpable disease (e.g. primary tumour, loco-regional recurrence) was measured and/or photographed wherever possible
8. If pulmonary disease was present patients had either a chest X-ray or a computed tomography (CT) scan of the thorax
9. If measurable liver disease was present the patient had an ultrasound or a contrast enhanced CT of the liver
  - If bone metastases were present, a radiograph of the involved bone(s) was obtained
10. Patients must have had at least 1 measurable lesion or an evaluable but not measurable lesion

In addition, the following was performed within 7 days prior to the date of starting treatment:

1. current medical conditions (includes conditions that are controlled by medication, and conditions related to previous chemotherapies)
2. concomitant therapy
3. physical examination (including WHO performance status, height, weight and vital signs)
4. haematology, biochemistry and blood sample
5. urine or serum pregnancy test in women of child-bearing potential (human chorionic gonadotrophic [HCG])

## **8.4 Tissue Collection**

Tumour tissue samples were collected at 4 separate time points on gefitinib (T0 – pre-treatment, T1 – after 8 weeks, T2 – after 6 months, T3 – at progression.)

Tissue was obtained via core cut needle biopsy in the clinic after the appropriate consent form had been signed. (Appendix 2) The core biopsy (x2) was placed in a labelled formalin pot and transferred to the pathology laboratory where it was fixed in 10% neutral buffered formalin for at least 24 hours. The sample was then catalogued and processed mechanically to provide a paraffin waxed metal cartridge. The sample was left to set then removed from the cartridge and placed in storage. The paraffin blocks were transported to the Tenovus laboratory for biomarker assessment. In addition a further core biopsy was snap frozen in liquid nitrogen and stored.

## 9 Biomarker Work

### 9.1 Background

All samples were immunocytochemically assayed and assessed in the Tenovus Centre for Cancer Research using standard operating procedures. All assays had been previously optimised and validated for use in paraffin-embedded clinical cancer material, including optimisation of antigen retrieval to maximally reveal each marker under test. The antibodies employed had previously been demonstrated extensively to be monospecific by Western blotting analysis, including employment of pharmacological challenge with specific signal transduction inhibitors *in vitro*. Furthermore, the immunohistochemical assay procedures employed had previously been demonstrated to be sensitive and specific using *in vitro* and /or clinical cancer preparations [132, 133, 158, 159, 176, 271-275]. Where phosphorylation / activation status was assessed, commercial purification by protein A and epitope-specific affinity chromatography was commonly used to ensure that the antibody was unable to detect the inactive form of the marker. Importantly, all phosphorylation sites chosen for analysis in the present study had previously been demonstrated to be key residues recruited during full activation of the signalling molecule under test. For all assays, matched sequential breast cancer samples for each anonymised patient were always run together, including an archived positive control paraffin-embedded breast cancer slide of known marker positivity for quality-control purposes. Buffer washes were performed between all primary antibody and detection steps (Table 3).

## **9.2 Assays and Procedures**

### **9.2.1 Antigen retrieval using microwaving in citric acid buffer**

#### **9.2.1.1 Demonstration of Ki67 or bcl2**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was carried out by microwaving sections in 0.01M citric acid buffer (pH 6) for 30 minutes at Power Level 6. Following non-specific blocking with 10% normal rabbit serum, slides were incubated with MIB-1 mouse anti-human Ki67 primary antibody (Dako) at 1/50 for 2 hrs or bcl2 (Clone 124; Dako) mouse anti-human primary antibody at 1/30 overnight at room temperature. This was followed sequentially by detection using “Super-Sensitive Link” (biotinylated anti-mouse immunoglobulins; Biogenex) and “Super-Sensitive Label” (streptavidin-peroxidase; Biogenex), each applied at 1/40 for 20 minutes. DAB was used as a chromogen. All slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

#### **9.2.1.2 Demonstration of HER2**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated. Methanol/hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was carried out by microwaving sections in 0.01M citric acid (pH 6) for 30 minutes at Power Level 6. Following non-specific blocking with 5% normal goat/human serum, slides were incubated with HER2 rabbit anti-human polyclonal antibody (Dako) at 1/300 for 2 hrs at room temperature. This was followed by detection

using goat anti-rabbit peroxidase-labelled IgG (Sigma-Aldrich) at 1/50 for 60min at room temperature and DAB chromogen. All slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

### **9.2.1.3 Demonstration of pMAPKinase**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was carried out by microwaving sections in 0.01M citric acid buffer (pH 6) for 30 minutes at Power Level 6. Following non-specific blocking with 20% normal human serum, slides were incubated with dually-phosphorylated erk1/2 MAP Kinase rabbit anti-human polyclonal antibody (Cell Signalling Technology) at 1/20 overnight at room temperature. This was followed by detection using “Multi-Link” (biotinylated anti-multi immunoglobulins; Biogenex) followed by “Concentrated Label” (streptavidin-peroxidase; Biogenex), each applied at 1/100 for 60 minutes. Following exposure to DAB chromogen, all slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

## **9.2.2 Antigen retrieval using microwaving in sodium citrate buffer**

### **9.2.2.1 Demonstration of pAKT**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was carried out by microwaving sections in 0.01M sodium citrate buffer (pH 6) for 1 min at Power Level 10 plus 9min at Power Level 6. Following non-specific blocking with 5% normal goat serum, slides were incubated with phosphorylated AKT rabbit polyclonal antibody (Cell Signalling Technology) at 1/50 overnight at room temperature. Detection subsequently employed rabbit-specific EnVision peroxidase-labelled polymer (Dako). Following exposure to DAB chromogen, all slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

## **9.2.3 Antigen retrieval using microwaving in EDTA**

### **9.2.3.1 Demonstration of pEGFR**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried briefly and stored at 4°C for activated EGFR. Before assay, sections were equilibrated to room temperature, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. Antigen retrieval was carried out by microwaving sections in EDTA (10mM; pH 8) for 1 minute on Full Power plus 9 minutes at Power Level 6. Following non-specific blocking with 0.02% PBS/Tween, slides were incubated with phosphorylation-specific mouse anti-human EGFR (Tyr1173) primary antibody (Chemicon) at 1/25 overnight at room temperature. Detection



employed mouse-specific EnVision peroxidase-labelled polymer and EnVision DAB chromogen (Dako). All slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

## **9.2.4 Antigen retrieval using enzymatic procedures**

### **9.2.4.1 Demonstration of EGFR**

5 micrometre sections of each formalin-fixed, paraffin-embedded breast cancer sample were dried, dewaxed and rehydrated, followed by a hydrogen peroxide endogenous peroxidase blocking step. Sections were exposed to 0.02% pronase E (Sigma-Aldrich) at 37°C for 30mins. Following a non-specific blocking step with 5% BSA/PBS, slides were incubated with mouse anti-human EGFR antibody (Clone 111.6; Neomarkers, 1/60) overnight at room temperature. This was followed sequentially by detection using “Super-Sensitive Link” (biotinylated anti-mouse immunoglobulins; Biogenex) and “Super-Sensitive Label” (streptavidin-peroxidase; Biogenex), each applied at 1/50 for 30 minutes. DAB was used as a chromogen. All slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

### **9.2.4.2 Demonstration of IGFR**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated followed by a hydrogen peroxide endogenous peroxidase blocking step. Sections were exposed to 0.02% pronase E (Sigma-Aldrich) at 37°C for 20mins. Following a non-specific blocking step with 5% BSA/PBS, slides were incubated with IGF1-R rabbit anti-human polyclonal antibody (Santa-Cruz) at 1/350 overnight at room temperature. Detection subsequently employed rabbit-specific EnVision peroxidase-labelled

polymer (Dako) for 2 hrs. Following exposure to DAB chromogen, all slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

### **9.2.5 Antigen retrieval using pressure cooking in sodium citrate buffer**

#### **9.2.5.1 Demonstration of oestrogen receptor alpha (ER ID5) and progesterone receptor (PgR 636)**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. For steam retrieval, sections were placed in a pressure cooker in 0.01M sodium citrate buffer (pH 6) for 2 minutes at full pressure. Following non-specific blocking with 20% normal human serum, slides were incubated with either ID5 (Dako) mouse anti-human ER alpha primary antibody or PgR636 (Dako) mouse anti-human PgR primary antibody at 1/300 for 60 minutes at room temperature. Detection employed a biotinylated goat anti-mouse/rabbit immunoglobulins solution (Dako; “Duet” kit) followed by streptABComplex/HRP (Dako; “Duet” kit) applied at 1/350 for 30 minutes. DAB was used as a chromogen. All slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

#### **9.2.5.2 Demonstration of pSER167 ER alpha, pSer118 ER alpha and pHER2**

5 micrometre sections of each formalin-fixed, paraffin-embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. For steam retrieval, sections were placed in a pressure cooker in 0.01M sodium citrate buffer (pH 6) for 2 minutes at full

pressure. Following blocking of non-specific binding, sections were incubated with activated Ser167 oestrogen receptor alpha (Cell Signalling Technology; 1/25), activated Ser118 oestrogen receptor alpha (Cell Signalling Technology; 1/25), or activated HER2 (Upstate; 1/250) rabbit anti-human polyclonal antibodies overnight at room temperature. Detection subsequently employed rabbit-specific EnVision peroxidase-labelled polymer (Dako). Following exposure to DAB chromogen, all slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

### **9.2.5.3 Demonstration of cfos**

5 micrometre sections of each formalin-fixed, paraffin-embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. For steam retrieval, sections were placed in a pressure cooker in 0.01M sodium citrate buffer (pH 6) for 2 minutes at full pressure. Following blocking of non-specific binding, sections were incubated with cfos (Santa Cruz; 1/100) rabbit anti-human polyclonal antibody overnight at room temperature. Detection employed “Multi-Link” (biotinylated anti-multi immunoglobulins; Biogenex) followed by “Concentrated Label” (streptavidin-peroxidase; Biogenex) at 1/100 for 30 minutes. Following exposure to DAB chromogen, all slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

## **9.2.6 Antigen retrieval using pressure cooking in EDTA buffer**

### **9.2.6.1 Demonstration of pIGFR**

5 micrometre sections of each formalin-fixed, paraffin embedded breast cancer sample were dried, dewaxed and rehydrated. Hydrogen peroxide was used to block endogenous peroxidase. For steam retrieval, sections were placed in a pressure cooker in EDTA (10mM; pH 8) for 6 minutes. Following non-specific blocking, slides were incubated with activated IGF1-R (Tyr1131)/Insulin Receptor (Tyr1146) rabbit anti-human polyclonal primary antibody (Cell Signalling Technology) at 1/10 overnight at room temperature. Detection employed rabbit-specific EnVision peroxidase-labelled polymer and DAB chromogen (Dako). All slides were counterstained using 0.5% methyl green, dehydrated and coverslipped prior to assessment.

Marker	Antigen retrieval	Blocking step	Antibody and source	Primary antibody dilution	Secondary detection system
ER	Pressure cook in pH 6 0.01M sodium citrate buffer (2 mins)	20% normal human serum in PBS	1D5 (#M7047) mouse antihuman ER monoclonal (Dako)	1/300 diluted in 20% normal human serum in PBS, 60 mins	Biotinylated goat anti-mouse/rabbit followed by StreptABComplex/HRP both at 1/350 diluted in 20% normal human serum/PBS, 30mins (Dako Duet kit)
PgR	Pressure cook in pH 6 0.01M sodium citrate buffer (2 mins)	20% normal human serum in PBS	PgR 636 (#M3569) mouse antihuman PgR monoclonal (Dako)	1/1000 diluted in PBS, 60 mins	Biotinylated goat anti-mouse/rabbit followed by StreptABComplex/HRP both at 1/350 diluted in 20% normal human serum/PBS, 30mins (Dako Duet kit)
EGFR	Protease P6911 (Sigma; 0.02% in 0.01M PBS [pH 7.2-7.4] at 37°C, 30 mins)	5% BSA in PBS	Mouse anti-EGFR monoclonal (Neomarkers, clone Ab-10 111.6; #MS378-P)	1/60 diluted in PBS, overnight	Biotinylated anti-immunoglobulin for mouse ('Link'), followed by streptavidin peroxidase kit ('Label') each at diluted 1/50 in 1% BSA/PBS, 30 mins (Biogenex)
HER2	Microwave in pH 6 0.01M citrate buffer 30 mins @ 560W	5% normal goat/normal human serum in PBS	Rabbit erbB2 antibody #A0485 (Dako)	1/300 diluted in 5% normal goat/normal human serum in PBS, 120 mins	Goat anti-rabbit IgG peroxidase conjugate (A4914 Sigma, 1/50 diluted in 5% normal goat/normal human serum in PBS), 60 mins
pEGFR	Microwave in 0.01M EDTA pH 8, 1min @full power+ 9 mins@560W	0.02% Tween/PBS	Mouse anti-tyrosine-phosphorylated EGFR monoclonal (#MAB3052; Chemicon)	1/45 diluted in PBS, overnight	Mouse EnVision peroxidase-labelled- polymer antibody (Dako) 120 mins
pHER2	Pressure cook in pH 6 0.01M sodium citrate buffer (2 mins)	1% BSA in PBS	Rabbit anti-activated erbB2 (Tyr1248 site) polyclonal (#06-229 Upstate)	1/250 diluted in PBS, overnight	Rabbit EnVision peroxidase-labelled-polymer antibody (Dako), 120 mins

Marker	Antigen retrieval	Blocking step	Antibody and source	Primary antibody dilution	Secondary detection system
pMAPK	Microwave in pH 6 0.01M citrate buffer 30 mins @560W	20% normal human serum in PBS	Rabbit anti-activated (dually phosphorylated Thr202/Tyr204) erk1/2 MAPK polyclonal (#9101, CST)	1/20 diluted in 20% normal human serum in PBS, overnight	“Multi Link” biotinylated anti-immunoglobulins followed by Concentrated “Label” Streptavidin Peroxidase both at 1/100 diluted in 1%BSA/PBS, 60 mins (Biogenex)
pAKT	Microwave in pH 6 0.01M sodium citrate buffer 1min @full power+ 9 mins @560W	5% normal goat serum in PBS	Rabbit anti-activated AKT polyclonal Ser473 site (#9277, CST)	1/50 diluted in PBS, overnight	Rabbit EnVision peroxidase-labelled-polymer antibody, 120 mins
IGFR	Protease P6911 (Sigma; 0.02% in 0.01M PBS [pH 7.2-7.4] at 37°C, 20 mins	5% BSA in PBS	Rabbit anti-IGFR polyclonal (#sc-712, SantaCruz)	1/350 diluted in PBS, overnight	Rabbit EnVision peroxidase-labelled-polymer antibody, 120 mins
pIGFR	Pressure cook in pH 6 0.01M EDTA buffer (6 mins)	0.02% Tween in PBS	Rabbit anti-activated IGFR Tyr1131/IR Tyr 1146 site polyclonal (#3021, CST)	1/10 diluted in PBS, overnight	Rabbit EnVision peroxidase-labelled-polymer antibody, 120 mins
Ki67	Microwave in pH 6 0.01M citrate buffer 30 mins @ 560W	10% normal rabbit serum in 0.1% BSA/PBS	MIB1 (#M7240 ) mouse monoclonal (Dako)	1/50 diluted in 0.1% BSA/ PBS, 120 mins	Biotinylated anti-immunoglobulin for mouse (‘Link’), followed by streptavidin peroxidase kit (‘Label’) each 1/40 diluted in 1%BSA/PBS, 20 mins (Biogenex)

Table 3: Biomarker Methodology: BSA, bovine serum albumin; EDTA, ethylenediamine tetra-acetic acid; HRP, horse-radish peroxidase

### **9.3 Assessment of Immunostaining**

Immunocytochemical analysis was performed in a blinded fashion (i.e. without knowledge of the patient clinical information). Samples were assessed using a standard operating procedure, examining all samples sequentially for a particular patient. Tumour epithelial cell immunopositivity appeared clearly as a brown nuclear signal against a background of green-blue nuclear counterstain. Any stromal cell immunostaining was not considered in this assessment. Immunostaining was: nuclear for ER alpha, PgR, cfos and Ki67; cytoplasmic for bcl2; nuclear and cytoplasmic for pMAPK and pSer118 ER alpha; plasma membrane and cytoplasmic for EGFR, pEGFR, HER2, pHER2, IGFR and pIGFR; nuclear, cytoplasmic and plasma membrane for pAKT and pSER167 ER alpha. Two experienced observers simultaneously assessed percentage tumour epithelial cell staining in each intensity category (i.e. negative, very weak +/-, weak +, moderate ++ and strong +++) using a dual-viewing attachment to a light microscope (BH-2; Olympus Optical Co., Germany). An overall examination of tumour epithelial immunostaining was first performed at an ocular magnification of x10 in order to avoid any associated normal/benign structures and to locate representative areas of tumour for further analysis. These areas were then viewed at x40 for more detailed tumour cell immunostaining assessment. Percentage positivity and staining intensity were assessed in several optical fields chosen at random, and a consensus figure for the whole slide was ascertained. This was performed in order to assign an HScore value for every breast cancer specimen, where the HScore is a well-established immunostaining index measured on a 0-300 scale. A total H Score was presented for each biomarker. For Ki67 immunostaining,

counting of percentage positivity only indicated cells in cycle vs. negative (G<sub>0</sub>) cells. Any samples with unacceptable levels of background staining, insufficient tumour material, or very poor or equivocal histological structure were eliminated. The positive control sections of known marker positivity were monitored to ensure adequate assay performance.

## **9.4 Statistical Analysis**

The study required 27 eligible patients in each patient population, and at least 4 patients in each population to derive clinical benefit (14.8% observed rate; 95% CI 5.2 - 31.0%). This would achieve a one-sided significance level of 4.2% and a power of 80.7% to conclude that the clinical benefit rate was > 5% when it is 20%. Recruitment into each arm was to be discontinued if there was no clinical benefit among the first 14 eligible patients (this would provide a > 95% certainty that the clinical benefit rate was < 20%). All patients who enrolled and received  $\geq 1$  dose of study medication were included in the intention-to-treat analysis for safety and efficacy.

Using recognised UICC criteria, complete (CR), partial (PR) and static (SD) response patients are grouped together as those achieving clinical benefit (CB). These are compared to those patients with progressive disease (PD) at the 6 month assessment.

All statistical analysis was performed using a software package SPSS version 17. Kaplan Meier survival curves were used to illustrate and analyse TTP by median pre-treatment marker expression. Pre-treatment biomarker staining was analysed using a Mann Whitney U test to determine differences in mean marker expression between responders (CB) and non-responders (PD). This is represented graphically as dot plots. The dots shown may represent several



coincident dots. A Mann Whitney U test was used to analyse the differences in mean biomarker staining from baseline (T0) to 8 weeks (T1). The changes in biomarker staining over time periods are shown as line graphs. The lines shown may represent several coincident lines. A Bonferroni adjustment was applied to allow for repeated measures leading to very stringent p values. The numbers of samples involved at the remaining time periods became so small that it was not possible to derive any meaningful statistics and so the data is simply described. As only a proportion of the data could be formally analysed we looked at medians and means and found the data to be similar, for consistency of descriptive data presentation and to aid interpretation, means, mean differences and confidence intervals were shown. In all cases a value of  $p \leq 0.05$  was considered significant.

## 10 Results

### 10.1 *In Vivo* Xenografts

#### 10.1.1 EGFR Expression

The TAMR xenografts expressed high EGFR levels in line with their parental cell lines [132] (Figure 3), indicating that there had not been a significant phenotypic shift during the creation of the tumour nodules and that potential sensitivity to gefitinib should have been retained.

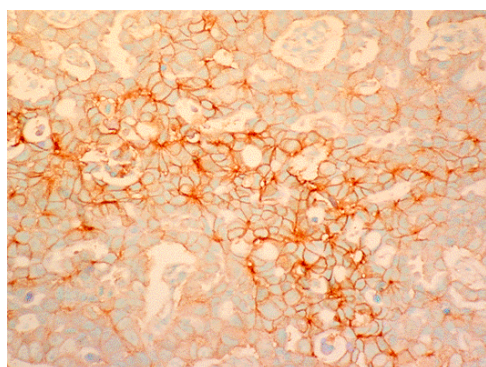


Figure 3: Tamoxifen Resistant Xenograft Expressing EGFR

#### 10.1.2 Growth Curves

In the clinical study, tamoxifen had been stopped once resistance had developed and prior to gefitinib administration, due to concerns regarding tamoxifen's potential agonistic effect in resistance. This raised the question as to how much of the gefitinib inhibitory growth effect in the ER-positive/TAMR patients might be due to tamoxifen withdrawal. In the *in vivo* experiment the mice were maintained on tamoxifen (as in the previous *in vitro* experiments [132, 133, 158]). The acquired TAMR xenografts treated with gefitinib in the presence of tamoxifen had lower mean levels of Ki67 than those maintained with tamoxifen alone (mean Ki67: 34.5% vs. 42.2%;  $p=0.068$ ) Examples of staining pre- and post-gefitinib treatment are shown in

Figures 4a and 4b. A random model coefficient demonstrated a statistically significant gefitinib repressive effect on tumour xenograft growth ( $p=0.039$ ; Figure 5 a - c). These data support the hypothesis that gefitinib is having an inhibitory effect on the xenografts and indicate that tumour shrinkage observed with administration of gefitinib *in vivo* is unlikely to be solely due to a tamoxifen-mediated withdrawal effect.

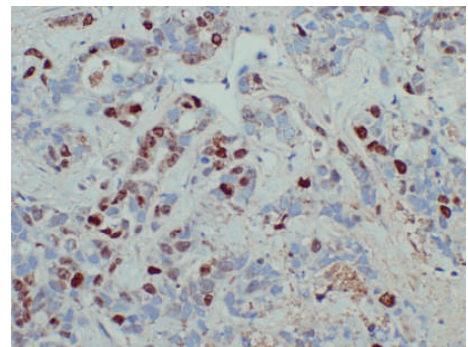
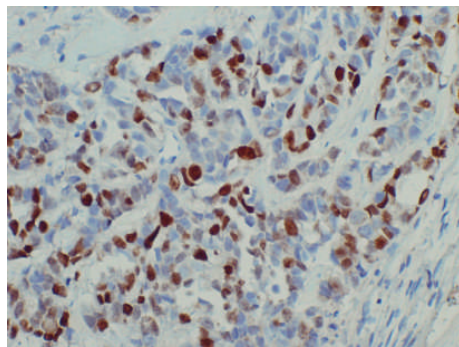


Figure 4a TAMR xenograft + Tamoxifen

Figure 4b TAMR xenograft +  
Tamoxifen & Gefitinib

Figure 4: Differences in Ki67 Staining in Treated Xenografts

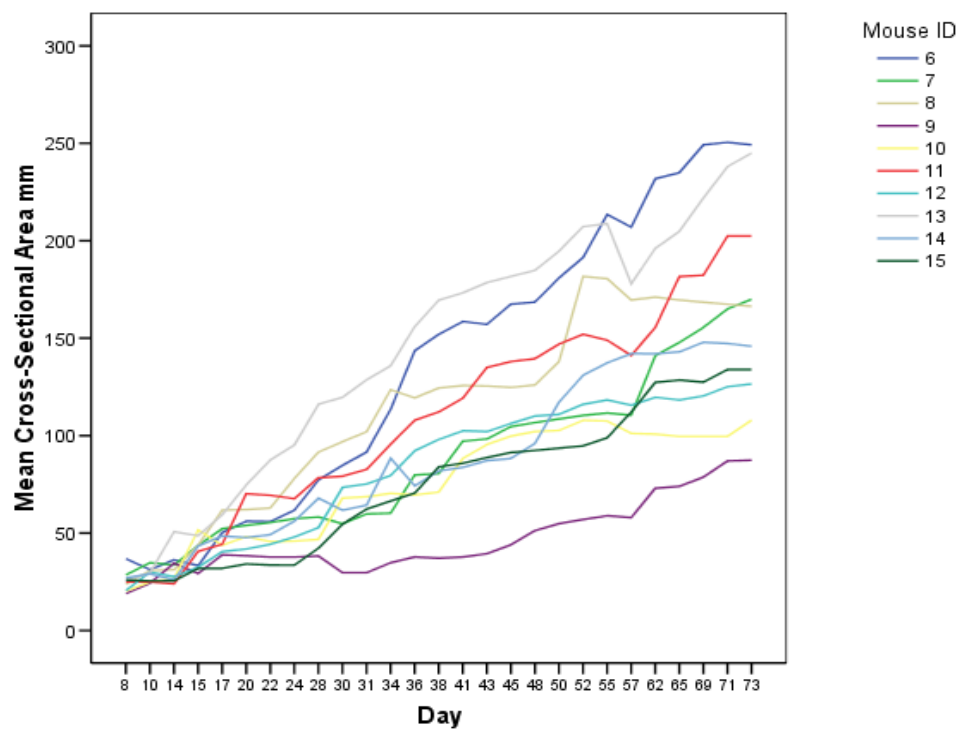


Figure 5a: Growth Curves for TAMR Xenografts Treated with Tamoxifen

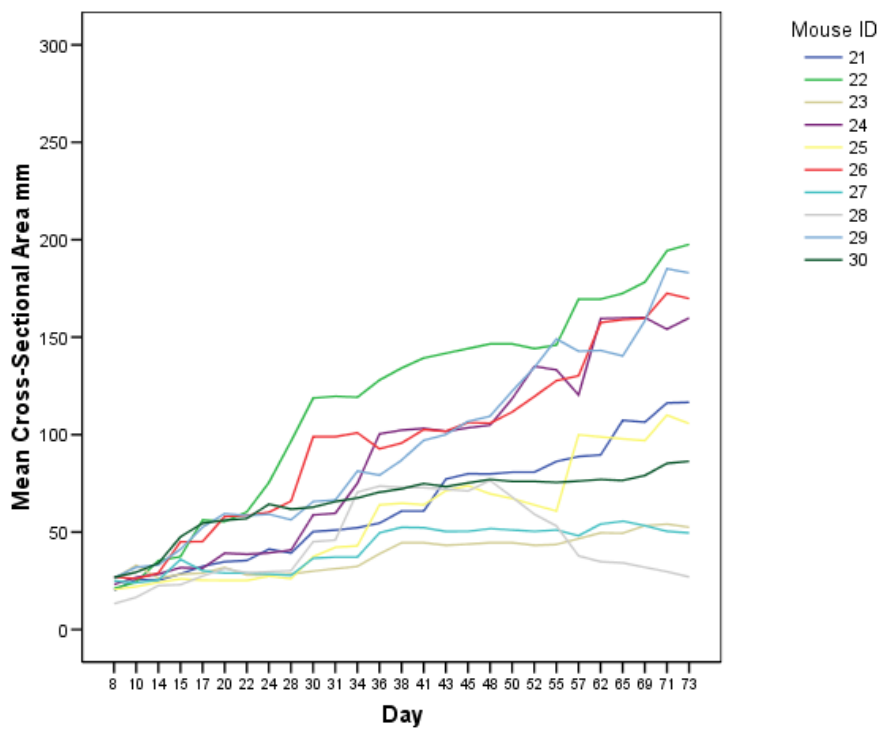


Figure 5b: Growth Curves for TAMR Xenografts Treated with Tamoxifen & Gefitinib

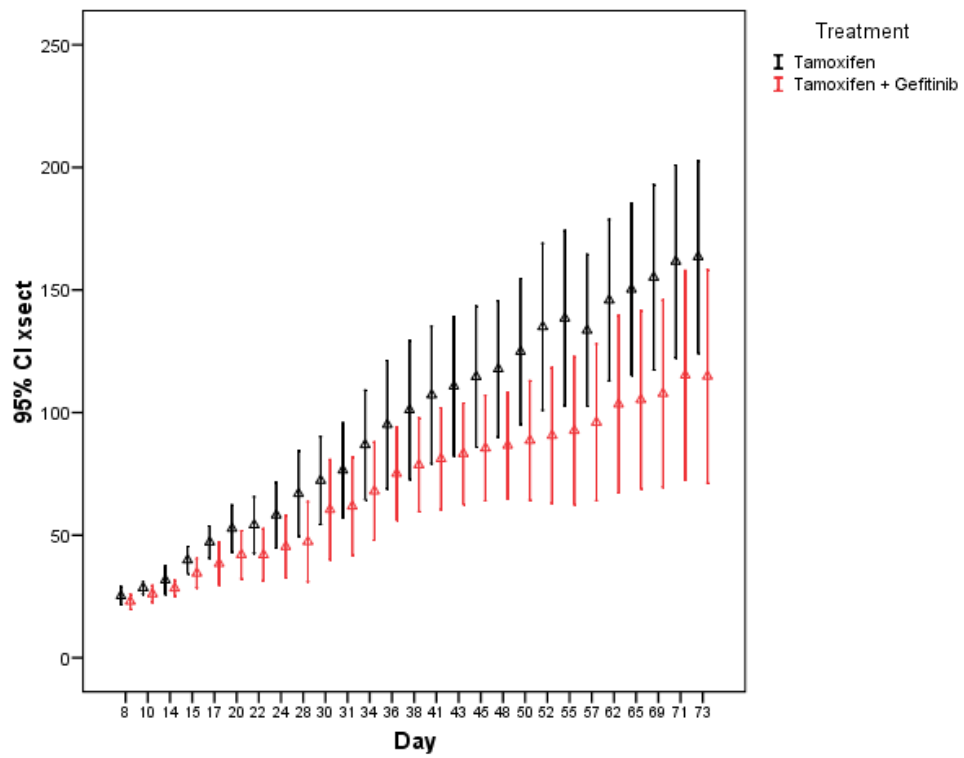


Figure 5c: Mean Growth Curves for the TAMR Xenografts by Treatment Group

## **10.2 Clinical Work**

### **10.2.1 Patient Characteristics**

A total of fifty-four patients (28 ER-positive TAMR and 26 ER-negative de novo resistant) were recruited between April 2001 and July 2005 (52 months) and the median follow up was 19.4 months (range, 1.32 – 65.33 months).

The median age was 61.5 years and the majority of patients (90.7%) had a WHO performance score of 0 or 1. Loco-regional disease was documented in 43% of all patients at presentation (19% had breast as their only site of disease) and 25%, 19%, and 17% of patients had bone, liver, and/or lung/pleural metastasis, respectively. Patients with ER-positive TAMR tumours (n=28) had a median age of 63 years (range, 42–82 years) and a median disease free interval (DFI) of 35.0 months (range, 7.8–296.2 months). There were a higher proportion of low grade tumours in this group. Patients with ER-negative tumours (n=26) had a median age of 61 years (range, 32–85 years) and DFI of 37.3 months (range 5.3–129.3 months), grade 3 tumours predominated (Table 4). In the ER-positive group 50% (n=14) had received tamoxifen as adjuvant therapy and 50% (n=14) had received tamoxifen as a first line therapy for metastatic/locally advanced disease.

<b>Demographic</b>	<b>ER-positive (n = 28)</b>	<b>ER-negative (n = 26)</b>
Age median (range)	63 (42-82)	61 (32-85)
WHO classification, n (%)		
0	14 (50)	11 (42.3)
1	14 (50)	10 (38.5)
2	0 (0)	5 (19.2)
DFI months median (range)	35 (7.8-296.2)	37.3 (5.3-129.3)
	95% CI 33.8-97.1	95% CI 28.7-60.6
Grade, n (%)		
1	0 (0)	0 (0)
2	10 (35.7)	3 (11.5)
3	7 (25.0)	18 (69.2)
Unassessable	11 (39.3)	5 (19.2)
Sites of disease, n (%)		
Breast alone	9 (17)	1 (2)
Breast	15 (53.6)	7 (26.9)
Skin	5 (17.9)	4 (15.4)
Bone	8 (28.6)	6 (23.1)
Liver	6 (21.4)	4 (15.4)
Lung	4 (14.3)	5 (19.2)
Nodes	11 (39.3)	14 (53.8)

Table 4: Demographics

### 10.2.2 Clinical Tolerability and Efficacy

Gefitinib treatment was generally well tolerated. The most common adverse events were in accordance with the known safety profile of gefitinib; dry skin or acneiform skin rash (n = 44, 81.5%), diarrhea (n = 34, 63%), nausea / vomiting (n = 21, 39%), lethargy (n=10, 19%) and alopecia (n=7, 13%). There

were 35 serious adverse events (five were drug-related) reported by 20 patients, and four deaths due to adverse events, none of which were related to therapy (bronchopneumonia, pleural effusion, constipation, and a cardiac arrest). There were no drug related significant clinical cardiac events and no patient required additional cardiac investigations whilst on trial. A total of 24 patients experienced a dose reduction to 250 mg and 8 patients were withdrawn due to adverse events, four of which were drug-related. (Table 5). There was no correlation between CTC grade of skin rash and response with ER negative patients displaying the most florid rashes. The most severe rash necessitating cessation of treatment occurred in an ER negative non-responder whilst the less severe rashes were commonly seen in ER positive responders.



<b>Adverse event</b>	<b>ER-positive (<i>n</i> = 28) <i>n</i> (%)</b>	<b>ER-negative (<i>n</i> = 26) <i>n</i> (%)</b>
Any AE	27 (96.4)	26 (100.0)
Drug-related AE	26 (92.9)	23 (88.5)
Serious AE	11 (39.3)	9 (34.6)
Serious drug-related AE	2 (7.1)	2 (7.7)
Withdrawal due to AE	5 (17.9)	3 (11.5)
Withdrawal due to drug- related AE	2 (7.1)	2 (7.7)
Withdrawal due to serious AE	3 (10.7)	2 (7.7)
Withdrawal due to serious drug-related AE	1 (3.6)	0 (0.0)
Death due to AE	2 (7.1)	2 (7.7)
Death due to drug-related AE	0 (0.0)	0 (0.0)
CTC grade 3 or 4	12 (42.9)	8 (30.8)
Drug-related CTC grade 3 or 4	4 (14.3)	4 (15.4)

Table 5: Gefitinib Tolerability

The overall clinical benefit rate was 33.3%. In the ER-negative patients the objective response rate and clinical benefit rate were 0% (n = 0) and 11.5% (n = 3), respectively, and median progression free survival was 1.84 months (range 0.66 – 8.45 months, 95% CI 1.54 – 2.37). In the ER-positive TAMR patients the objective response rate was 7.1% (n = 2; both PR, although one patient achieved a prolonged CR in her liver metastases whilst her bone disease was evaluable rather than measurable). The duration of response for these patients was prolonged at 45 and 13.8 months. The clinical benefit rate was 53.6% (n = 15), and the median progression free survival was 8.74 months (range 0.92 – 32.7, 95% CI 3.52 – 12.19). Median overall survival was prolonged in the ER-positive TAMR group compared with ER-negative group (32.56 versus 8.79 months, P = 0.001), as was post-gefitinib survival (21.12 versus 4.90 months, P = 0.002). (Table 6).

Patient subgroup	Clinical benefit, % (95% CI)	Overall tumor response, % (95% CI)	PFS in months Median (range) (95% CI)	Survival in months Median (range) (95% CI)	Post-gefitinib survival in months Median (range) ( 95% CI)
ER-positive (n = 28)	53.6 (n = 15) (95% CI 33.9-72.5)	7.1 (0.9-23.5) (n = 2)	8.74 (0.92-32.7) (95% CI 3.52-12.19)	32.56 (1.32-65.33) (95% CI 23.5-37)	21.12 (0-43.5) (95% CI 15-26.1)
ER-negative (n = 26)	11.5 (n = 3) (95% CI 2.4-30.2)	0 (0-13.2) (n = 0)	1.84 (0.66-8.45) (95% CI 1.54-2.37)	8.79 (1.35-44.8) (95% CI 7.9-18.4)	4.90 (0-35.6) (95% CI 4.8-13.3)

Table 6: Clinical Efficacy of Gefitinib

### **10.3 Tissue Samples**

Pre-treatment tissue samples were available for 38 patients (14 ER-positive/TAMR and 24 ER-negative). Of these n=10 were historical primary tumour samples with no subsequent biopsies, leaving a potential 28 sets of matched samples. Of these n=8 with-drew from the study due to side effects. This lead to n=15 matched pair samples for the time period T0 – T1, n=7 matched pair samples for the time period T0 – T2 and n=12 matched pair samples at progression (Figure 6). The missing samples are due to a combination of omitted biopsies, poor samples, problems with some of the assays and patients not having reached the relevant biopsy time points or having had their samples assayed at the time of analysis. Some progression biopsies were not taken due to patients withdrawing consent at this time point.

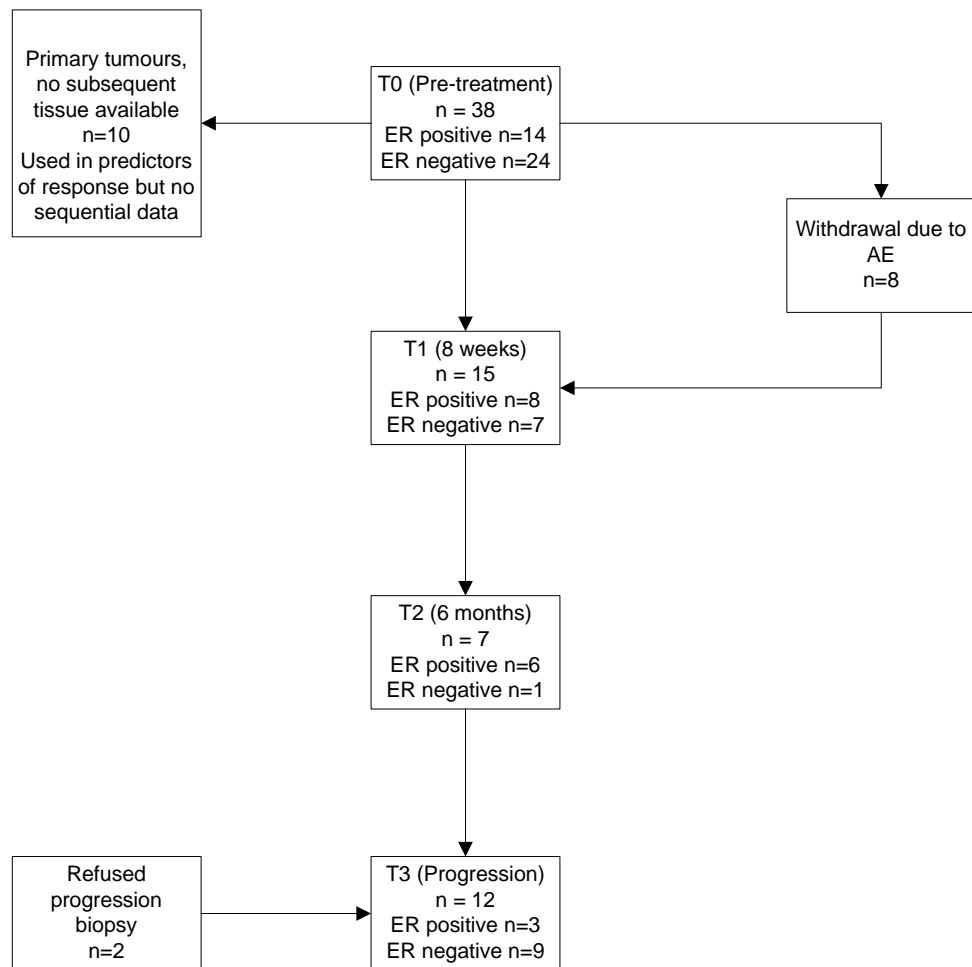


Figure 6: Consort Diagram for Biopsies

## **10.4 Predictors of Clinical Outcome**

Pre-treatment tissue samples were available for 38 patients (14 ER-positive/TAMR and 24 ER-negative). Baseline biomarker expression was examined for correlation with clinical benefit rate and disease progression (Table 7). The median H Score was used to define a cut-off for higher levels of positivity for each marker, with the exception of ER, where any staining was considered positive.

Using the sensitive immunodetection assay, all gefitinib responders (i.e., those achieving CB) expressed EGFR (median 30, range 10-65), although EGFR staining was more commonly weakly cytoplasmic in patients achieving CB, with more prominent plasma membrane-staining in patients with PD. High levels of EGFR expression were associated with PD ( $P = 0.005$  BF 0.075; Table 6). ER and PgR positivity predicted CB ( $P < 0.001$  BF 0.015 and 0.016 BF 0.24, respectively; Table 6). ER positivity was by far the strongest predictor of CB. In the ER-positive group, 66% of patients were dual positive for ER and PgR and achieved a CBR of 87.5%, compared with 75% observed in the ER-positive, PgR-negative group. Expression of pEGFR, HER2, pHER2, IGFR, pIGFR, Ki67, pMAPK, pAKT, bcl2, cfos, Ser167 and Ser118 in the pre-treatment samples did not relate to outcome.

<b>Biomarker</b>	<b>CB (n = 12) % highly positive</b>	<b>PD (n = 23) % highly positive</b>	<b>Median H score (range)</b>	<b>p value</b>	<b>Bonferroni Adjustment</b>
ER	81.8	13	5 (5-190)	0.001	<b>0.015</b>
PgR	58.3	15.4	55 (2-230)	0.016	0.24
EGFR	20	68.2	40 (2-230)	0.005	0.075
pEGFR	54.5	45.5	30 (5-109)	0.55	>1
HER2	50	50	90 (0-290)	0.65	>1
pHER2	60	50	130 (3-295)	0.44	>1
IGFR	50	52.2	130 (15-290)	0.59	>1
pIGFR	33.3	47.8	24.5 (0-220)	0.32	>1
Ki67	63.6	66.7	30 (1-85)	0.66	>1
pAKT	45.5	41.7	36 (3-200)	0.62	>1
pMAPK	41.7	63.6	90 (32-220)	0.19	>1
bcl2	66.7	30	87.5 (2-200)	0.46	>1
cfos	50	70	85 (10-130)	0.60	>1
Ser167	33.3	66.7	117 (50-280)	0.47	>1
Ser118	66.7	40	140 (32-240)	0.66	>1

Table 7: Predictors of Outcome

### ***10.5 Time to Progression***

ER and PGR positivity conferred a longer TTP. ER positive patients had a mean TTP of 9.5 months, range 3 - 15, the ER negative subgroup had a mean TTP of 2.9 months, range 1 – 7.75 (  $p=0.001$ , BF  $p=0.015$ ) (Figure 7). The PGR positive patients had a mean TTP of 9.15 months, range 4.75-12, the PGR negative subgroup had a mean TTP of 4.56 months, range 1 - 15 ( $p=0.01$ , BF  $p=0.15$ ) (Figure 8). High levels of EGFR expression were associated a shorter TTP 3 months, range 1-7.75, vs. 6.1 months, range 1-15 ( $p=0.01$ , BF  $p=0.15$ ) (Figure 9). For pEGFR, HER2, pHER2, Ki67, IGFR, pIGFR, pMAPK, pAKT bcl2, cfos, Ser167 and Ser118 there were no obvious differences seen in mean TTP between the designated positive and negative groups (Figures 10 – 20) (Table 8) In the case of Ser167 there seemed to be a separation of the curves with the high expressors progressing in 3.93 months vs. 7.11 months but the confidence intervals were wide and this did not reach significance.



Marker	Status	Mean TTP months (range)	95% Confidence Interval	p value	Bonferroni Adjustment
ER	Positive	9.5 (3-15)	6.5 – 12.50	0.001	<b>0.015</b>
	Negative	2.9 (1-7.75)	1.42 – 4.37		
PgR	Positive	9.15 (4.75-12)	5.38 – 12.93	0.01	0.15
	Negative	4.56 (1-15)	2.04 – 7.08		
EGFR	Positive	3 (1-7.75)	1.79 – 4.21	0.01	0.15
	Negative	6.1 (1-15)	2.89 – 9.29		
pEGFR	Positive	5.12 (1-15)	2.78 – 7.45	0.51	>1
	Negative	3.23 (1-9)	1.21 – 5.24		
HER2	Positive	5.23 (1-15)	2.79 – 7.67	0.33	>1
	Negative	3.25 (1-9)	1.29 – 5.22		
pHER2	Positive	4.84 (1 – 15)	2.29 – 7.39	0.57	>1
	Negative	3.75 (1 – 9)	1.83 – 5.67		
Ki67	Positive	6.15 (1-12)	3.10 – 9.20	0.64	>1
	Negative	5.43 (1.25 – 15)	1.49 – 9.38		
IGFR	Positive	3.46 (1-9)	1.74 – 5.19	0.40	>1
	Negative	5.5 (1.5 – 15)	2.49 – 8.50		
pIGFR	Positive	4.62 (1 – 12)	2.40 – 6.83	0.35	>1
	Negative	4.08 (1-15)	1.52 – 6.64		
pMAPK	Positive	3.32 (1-9)	1.82 – 4.81	0.15	>1
	Negative	5.93 (1-15)	2.56 – 9.30		
pAKT	Positive	5.13 (1-15)	2.41 – 7.86	0.99	>1
	Negative	3.52 (1-9)	1.78 – 5.26		
bcl2	Positive	7.13 (2-12)	3.59 – 10.66	0.17	>1
	Negative	4.8 (1-15)	1.66 – 7.94		
cfos	Positive	4.70 (1-9)	2.72 – 6.69	0.18	>1
	Negative	7.60 (1.25 – 15)	2.29 – 12.93		
pSer167	Positive	3.93 (1-8)	1.71-6.16	0.17	>1
	Negative	7.11 (1.25 - 15)	1.97 – 12.25		
pSer118	Positive	5.21 (1-15)	2.66 – 7.77	0.47	>1
	Negative	3.27 (1-9)	1.58 – 4.97		

Table 8: Time to Progression by Baseline Marker Expression

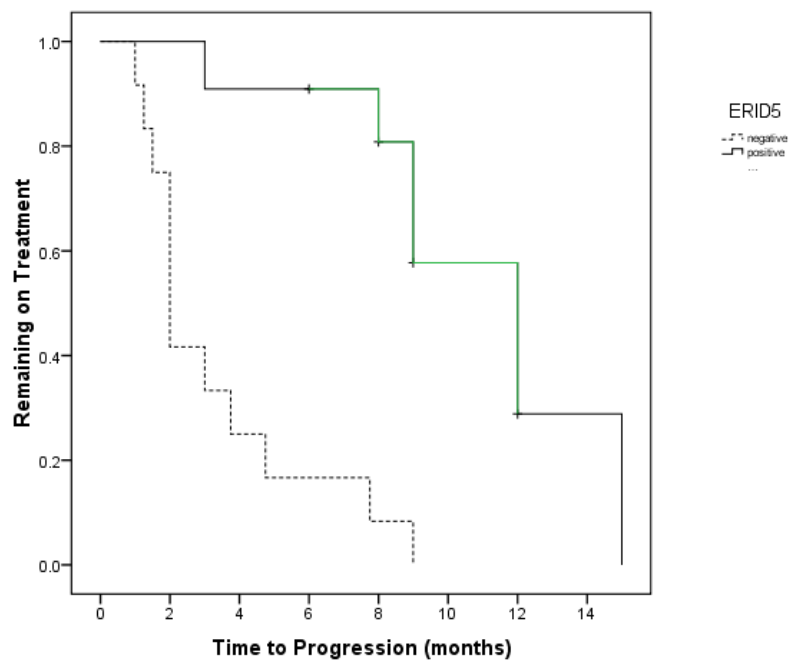


Figure 7: Time to Progression by ERID5 Status

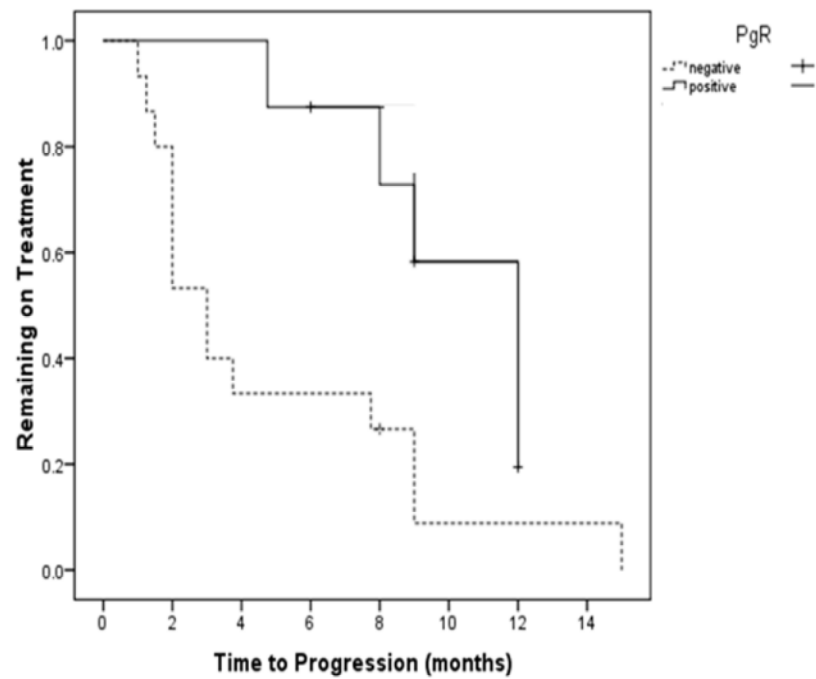


Figure 8: Time to Progression by PgR Status

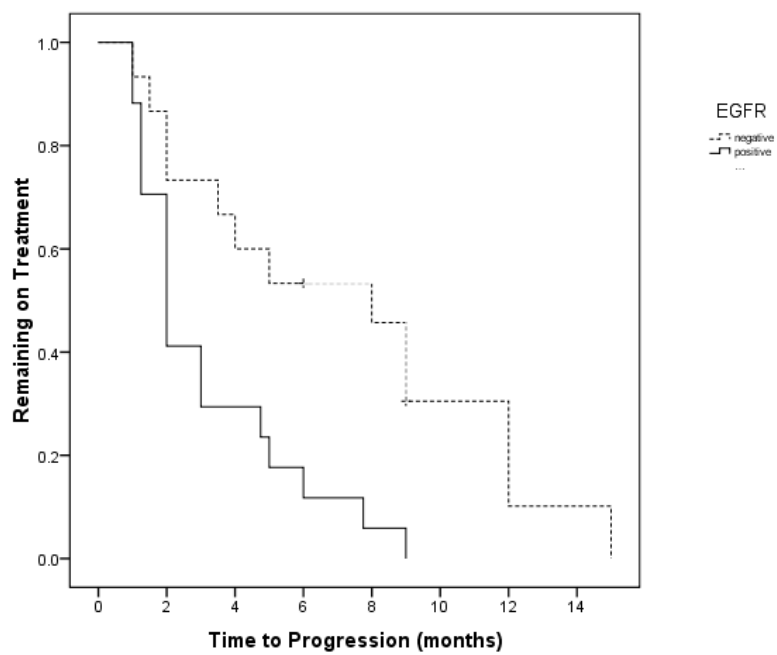


Figure 9: Time to Progression by EGFR Status

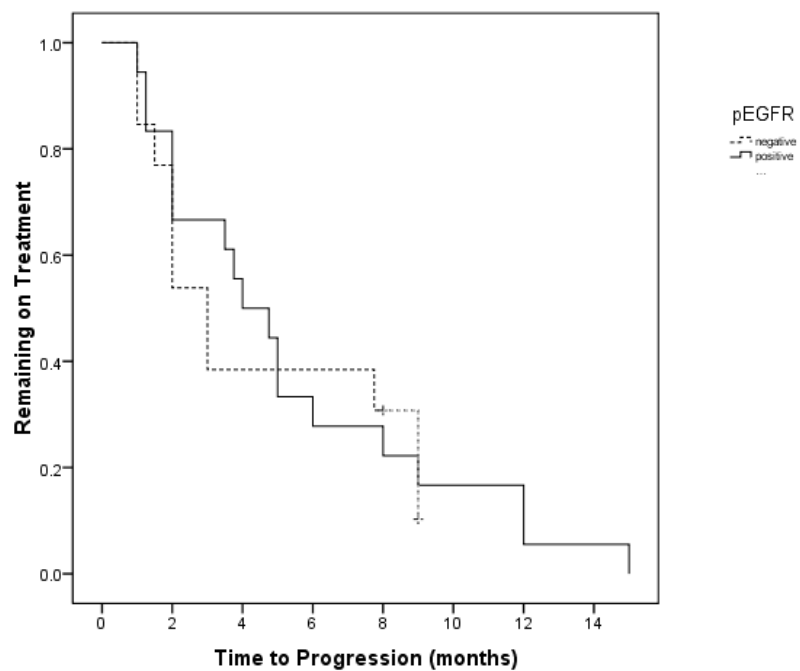


Figure 10: Time to Progression by pEGFR Status

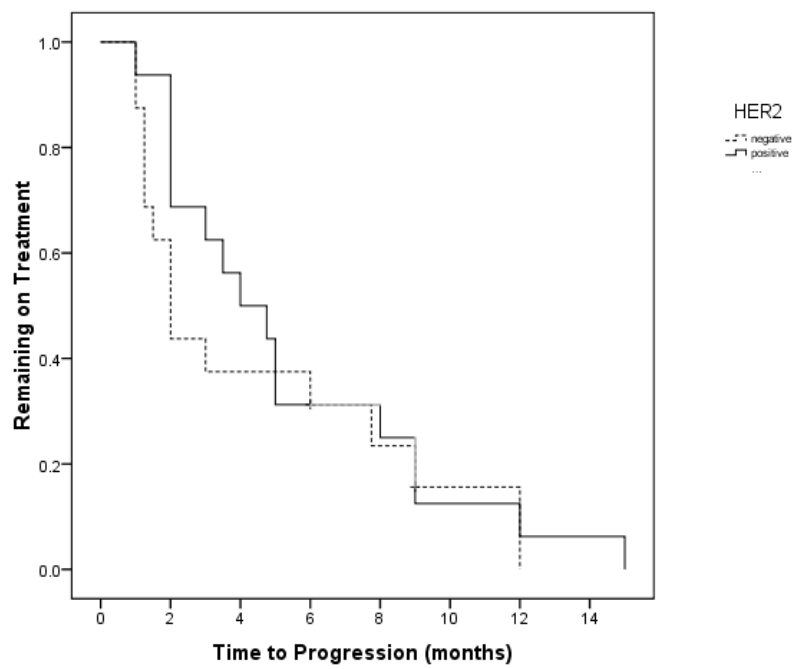


Figure 11: Time to Progression by HER2 Status

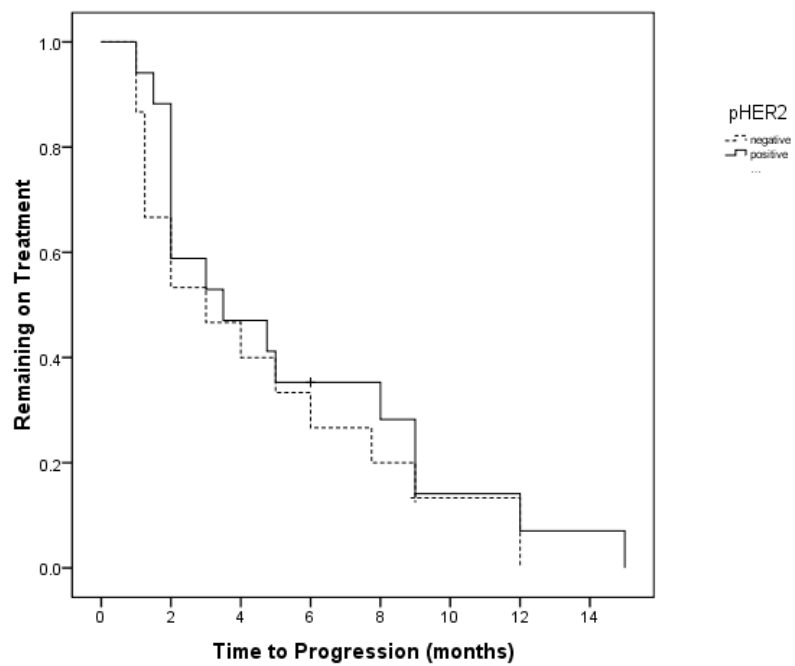


Figure 12: Time to Progression by pHER2 Status

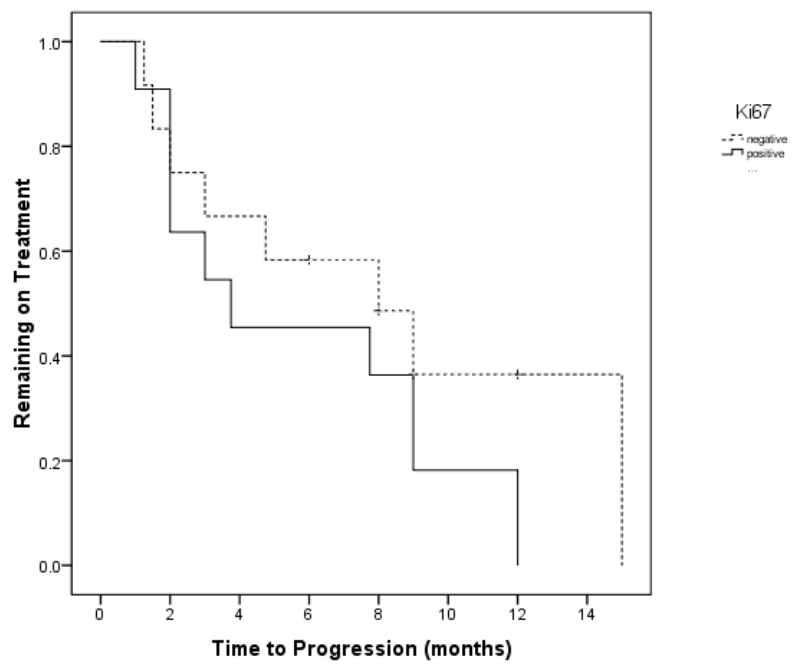


Figure 13: Time to Progression by Ki67 Status

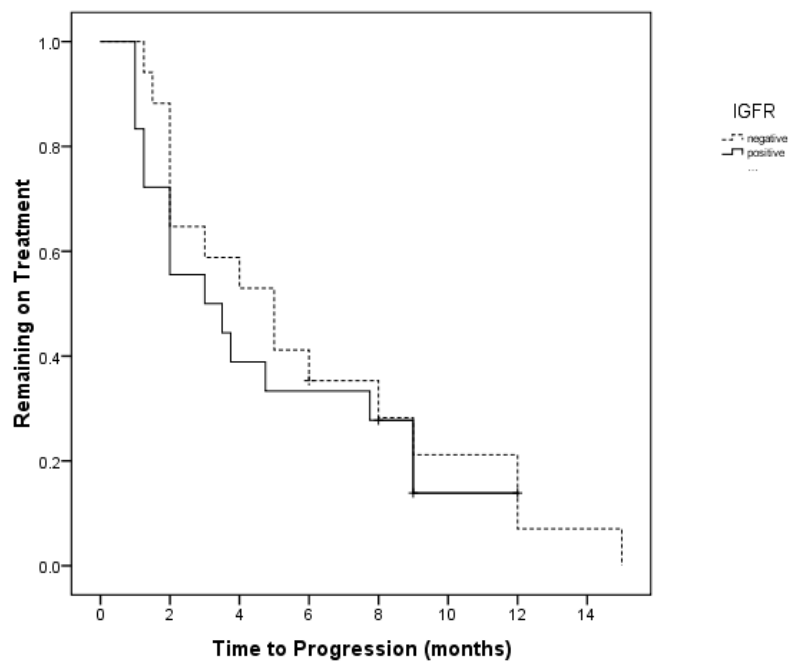


Figure 14: Time to Progression by IGFR Status

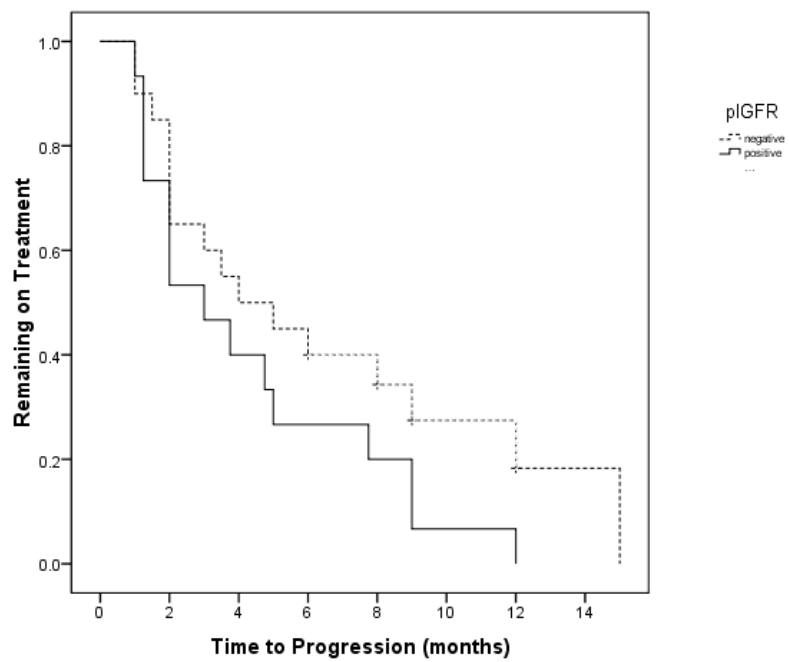


Figure 15: Time to Progression by pIGFR Status

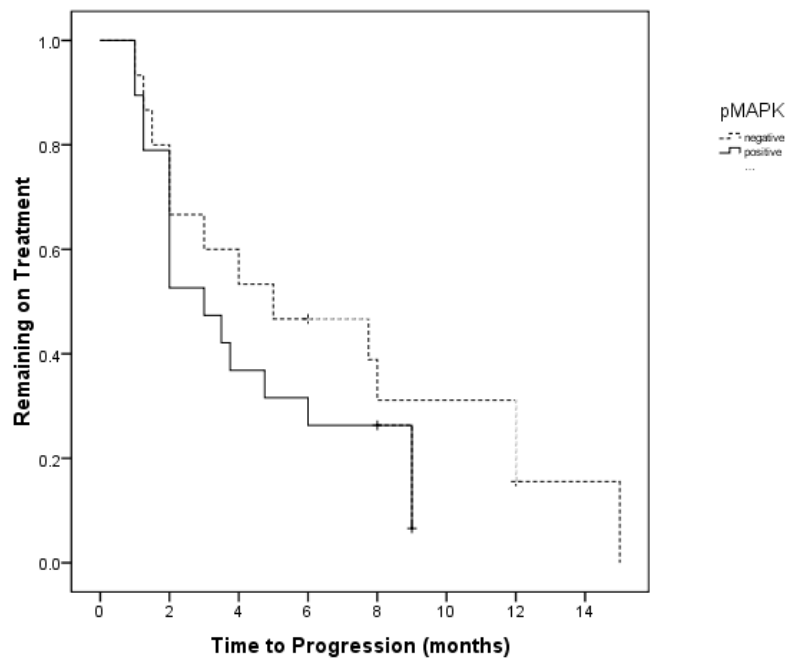


Figure 16: Time to Progression by pMAPK Status

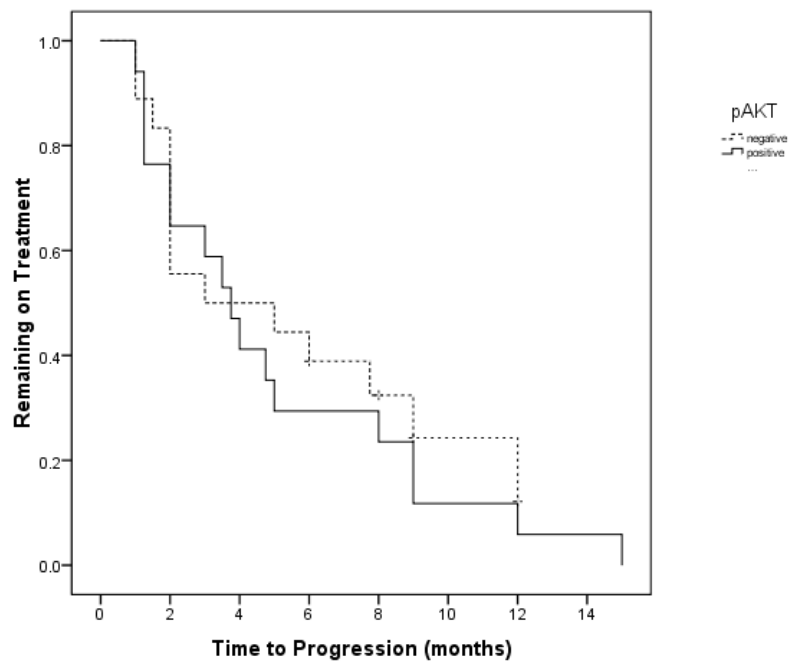


Figure 17: Time to Progression by pAKT Status

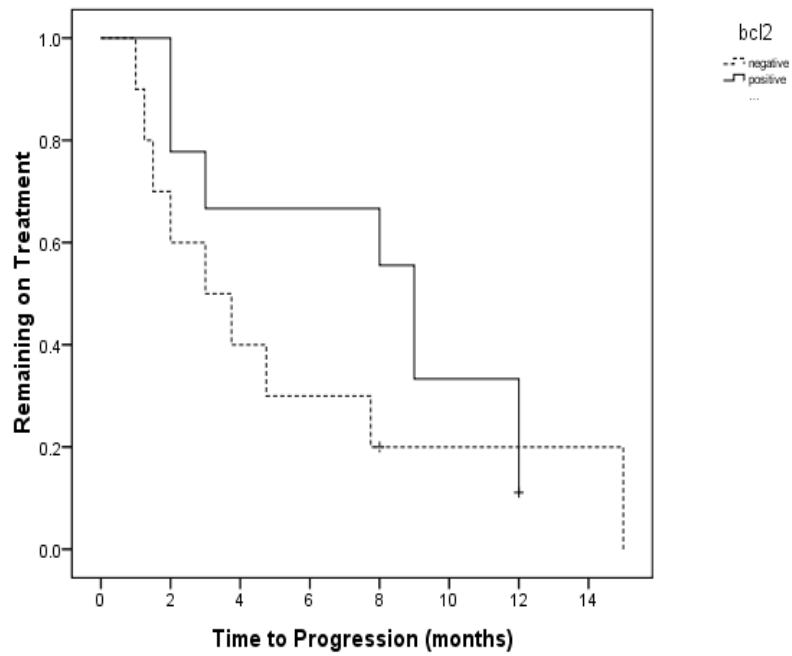


Figure 18: Time to Progression by bcl2 Status

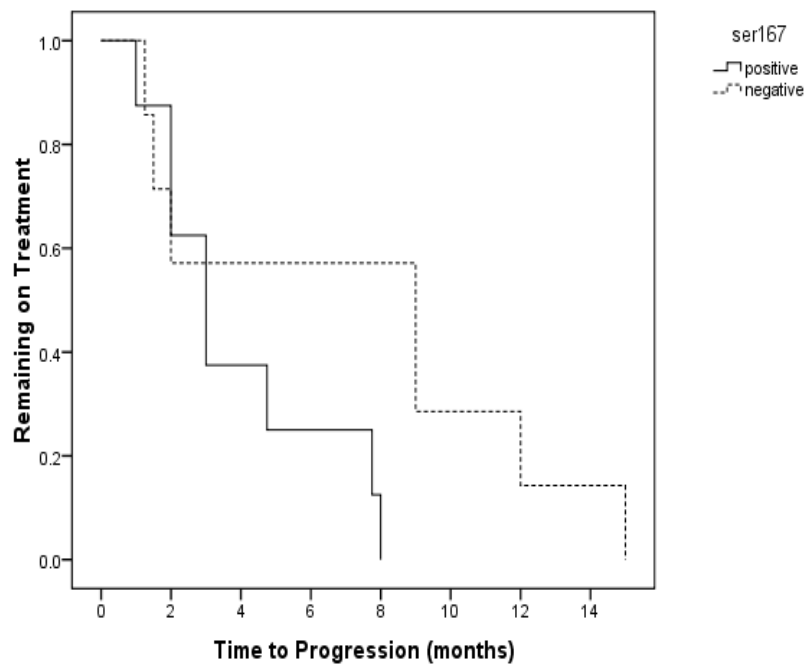


Figure 19: Time to Progression by pSer167 Status

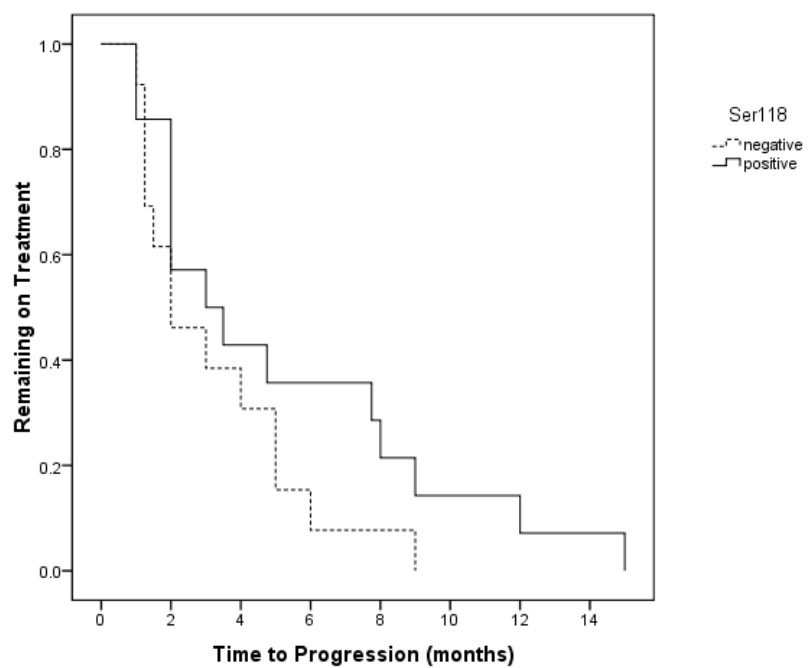


Figure 20: Time to Progression by pSer118 Status



## **10.6 Predictors of Response at T0**

ER positivity was by far the strongest predictor of clinical benefit (CB). The mean H Score was 100, range 0-175 in CB vs. 34.1, range 0 – 190 in PD ( $p=0.012$  BF 0.18) (Figure 21). PgR positivity alone was not so strongly associated with response. The mean H Score was 35, range 0-150 in CB vs. 21.6, range 0 – 230 in PD ( $p=0.287$  BF  $>1$ ) (Figure 22). However in the ER-positive group, 66% of patients were dual positive for ER and PgR and these patients achieved a clinical benefit rate (CBR) of 87.5%, compared with 75% observed in the ER-positive, PgR-negative group.

Higher levels of EGFR expression were seen with PD ( $p=0.121$  BF 0.075) (Figure 23)). The mean H Score in CB was 42.4, range 10 -130 vs. 72.8, range 5 – 230 in PD. All gefitinib responders expressed EGFR to some degree. The frequency of HER2 expression in the ER-negative tumours was, as expected, high (mean 130, range 45-235). In the ER-positive/TAMR tumours, HER2 expression levels were higher than would be expected in such relatively treatment naïve breast cancers (mean 92.5, range 15-195), but was in accordance with *in vitro* observations of increased HER2 expression in breast cancer cell lines with acquired TAM resistance [132]. Of note, high levels of HER2 in the ER-positive cohort did not preclude a response to gefitinib, and there was no association between HER2, or its activation and gefitinib response in the whole patient group. Equally, the expression or activity of IGFR in the pre-treatment samples did not predict response or failure although the PD group expressed higher mean levels of pIGFR (52.3 vs. 32.92) and pAKT (55.58 vs. 34.92) (Figures 24 – 35) (Table 9).

<b>Marker</b>	<b>HScore mean (range)</b>	<b>p value</b>	<b>Bonferroni Adjustment</b>
ER	<b>CB</b> 100 (0-175)	0.012	0.18
	<b>PD</b> 34.1 (0-190)		
PgR	<b>CB</b> 35 (0-150)	0.287	>1
	<b>PD</b> 21.6 (0-230)		
EGFR	<b>CB</b> 42.4 (10-130)	0.121	>1
	<b>PD</b> 72.8 (5-230)		
pEGFR	<b>CB</b> 44 (5-109)	0.529	>1
	<b>PD</b> 36.70 (5-90)		
HER2	<b>CB</b> 99.8 (0-195)	0.139	>1
	<b>PD</b> 131.8 (45-290)		
pHER2	<b>CB</b> 122.7 (3-280)	0.468	>1
	<b>PD</b> 154.7 (20-295)		
Ki67	<b>CB</b> 38.92 (8-85)	0.468	>1
	<b>PD</b> 30.84 (2-80)		
IGFR	<b>CB</b> 117.9 (65-160)	0.533	>1
	<b>PD</b> 116.9 (15-290)		
pIGFR	<b>CB</b> 32.92 (0-80)	0.649	>1
	<b>PD</b> 52.3 (0-180)		
pMAPK	<b>CB</b> 100.83 (36-205)	0.944	>1
	<b>PD</b> 103.68 (13-220)		
pAKT	<b>CB</b> 34.92 (10-80)	0.287	>1
	<b>PD</b> 55.58 (3-200)		
Bcl2	<b>CB</b> 97.33 (2-200)	0.216	>1
	<b>PD</b> 72.21 (10-180)		
Cfos	<b>CB</b> 71.58 (15-120)	0.929	>1
	<b>PD</b> 84.21 (10-180)		
pSer167	<b>CB</b> 108.75 (60-220)	0.462	>1
	<b>PD</b> 136.21 (20-280)		
pSer118	<b>CB</b> 100.21 (0-175)	0.180	>1
	<b>PD</b> 131.58 (0-190)		

Table 9: Predictors of Response at Pre-treatment (T0)

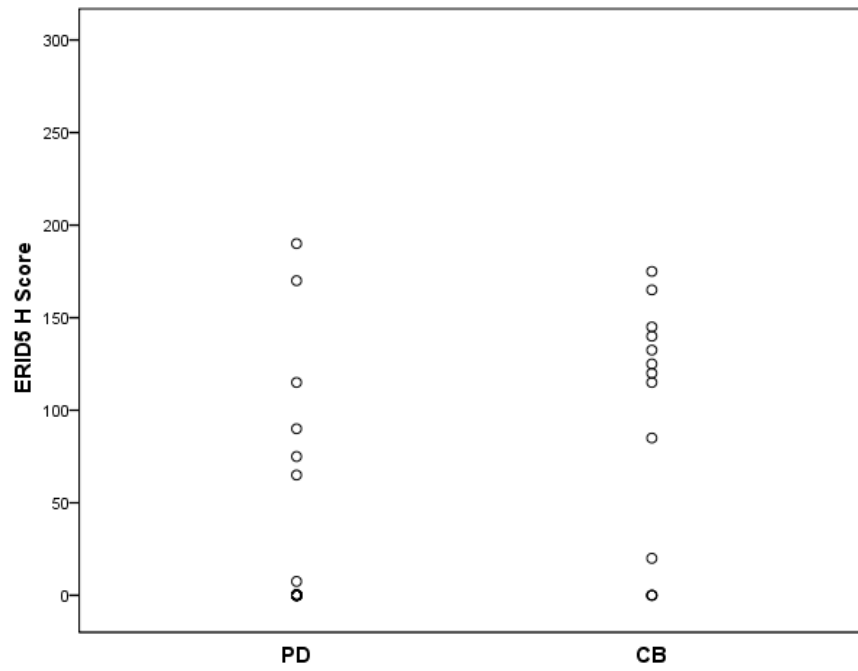


Figure 21: Pre-treatment (T0) ER Expression by Response

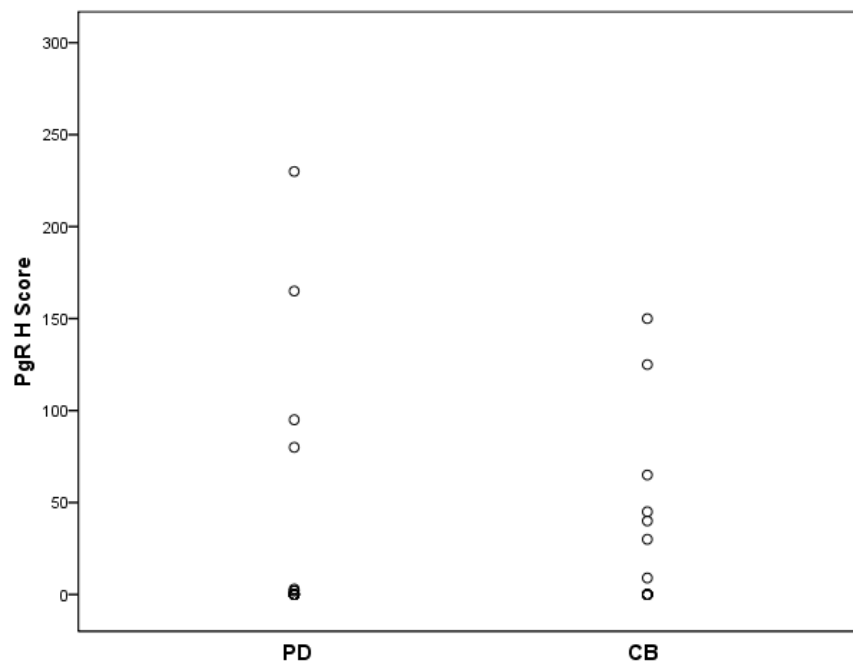


Figure 22: Pre-treatment (T0) PgR Expression by Response

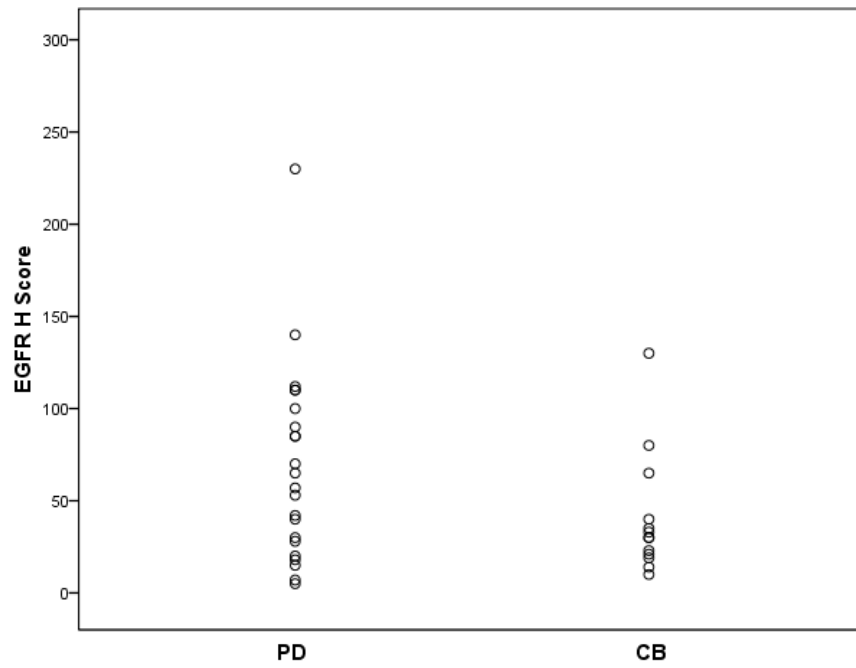


Figure 23: Pre-treatment (T0) EGFR Expression by Response

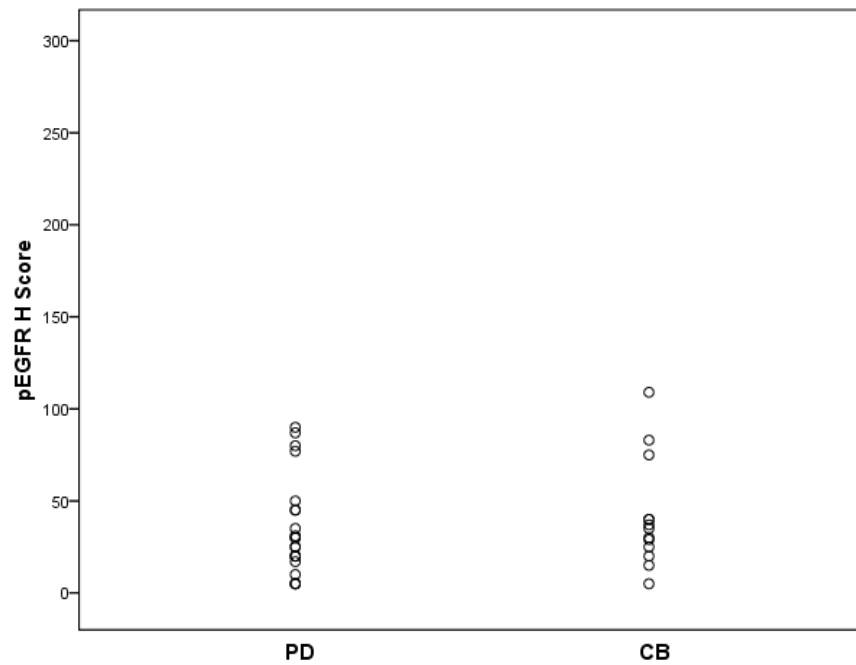


Figure 24: Pre-treatment (T0) pEGFR Expression by Response

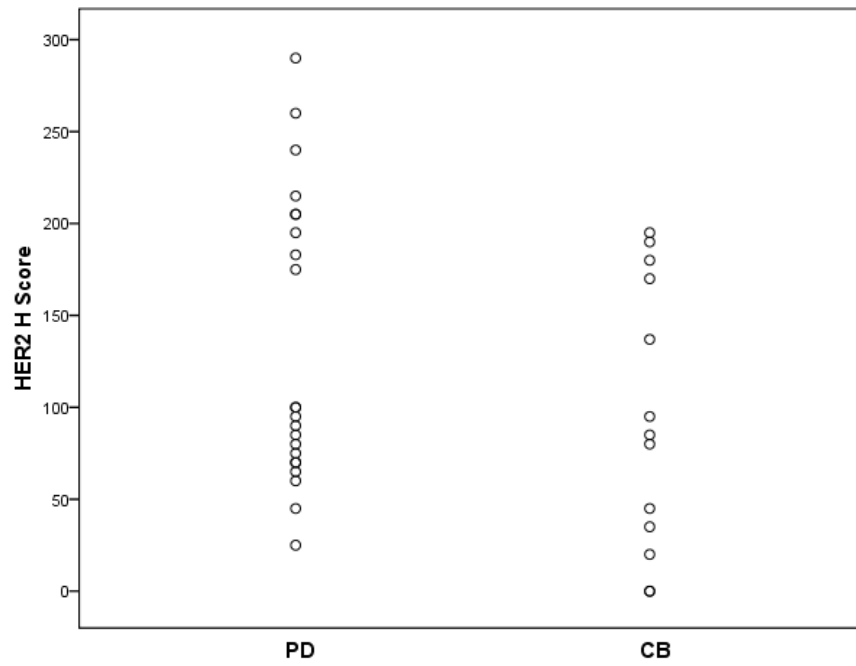


Figure 25: Pre-treatment (T0) HER2 Expression by Response

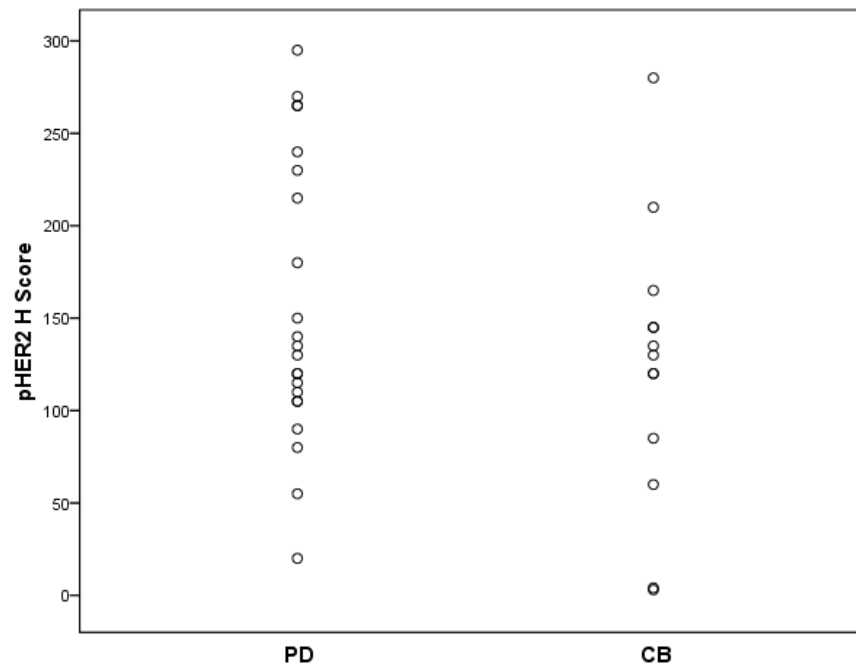


Figure 26: Pre-treatment (T0) pHER2 Expression by Response

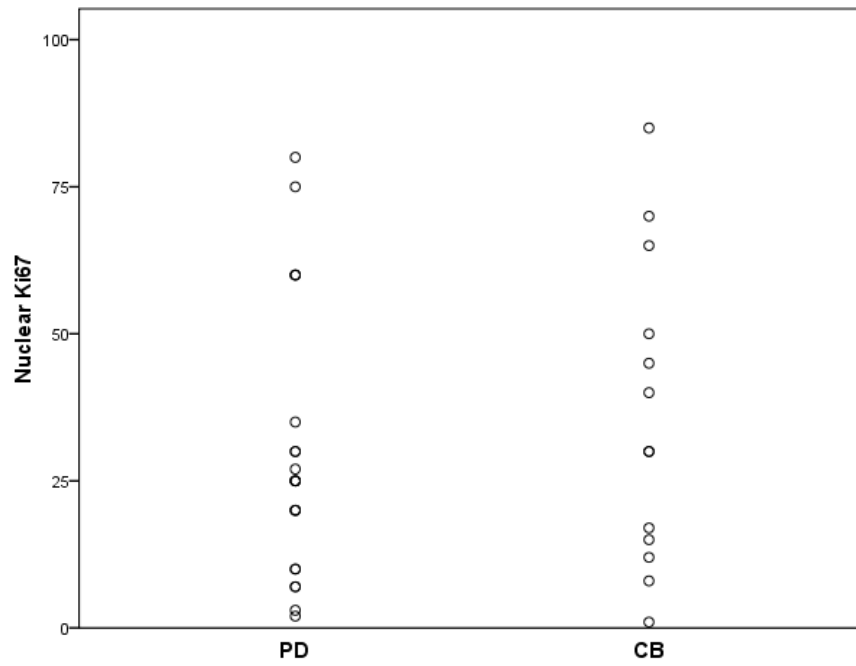


Figure 27: Pre-treatment (T0) Ki67 Expression by Response

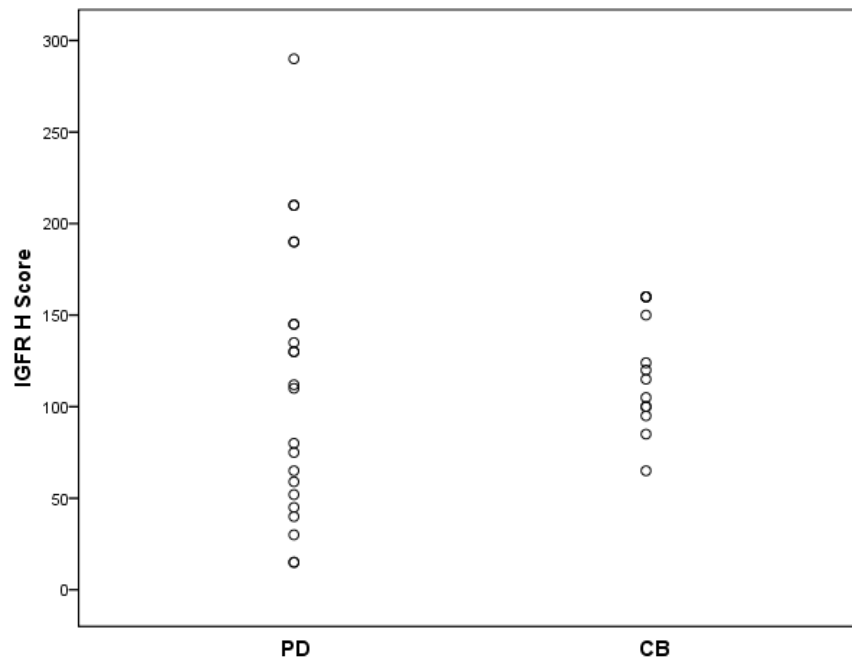


Figure 28: Pre-treatment (T0) IGFR Expression by Response

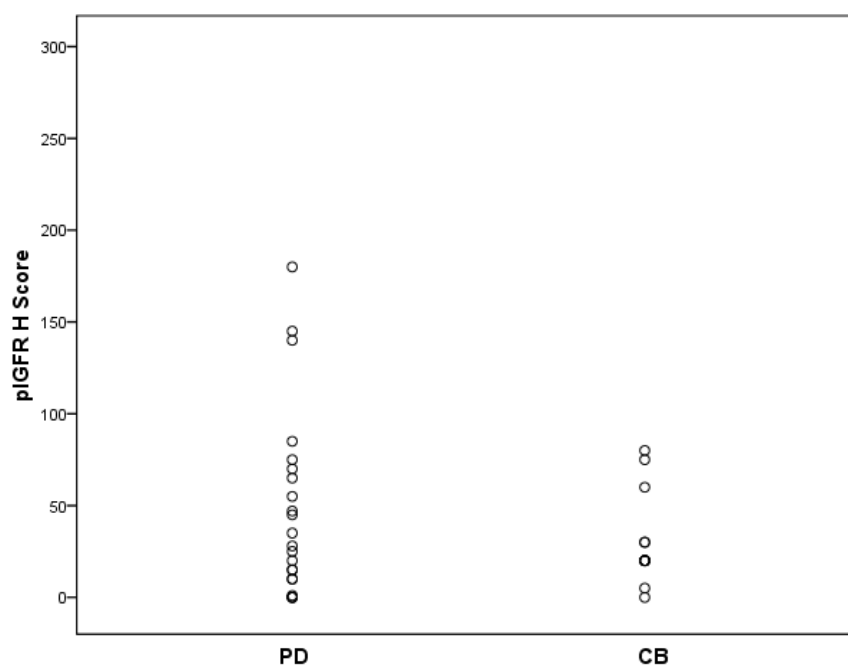


Figure 29: Pre-treatment (T0) pIGFR Expression by Response

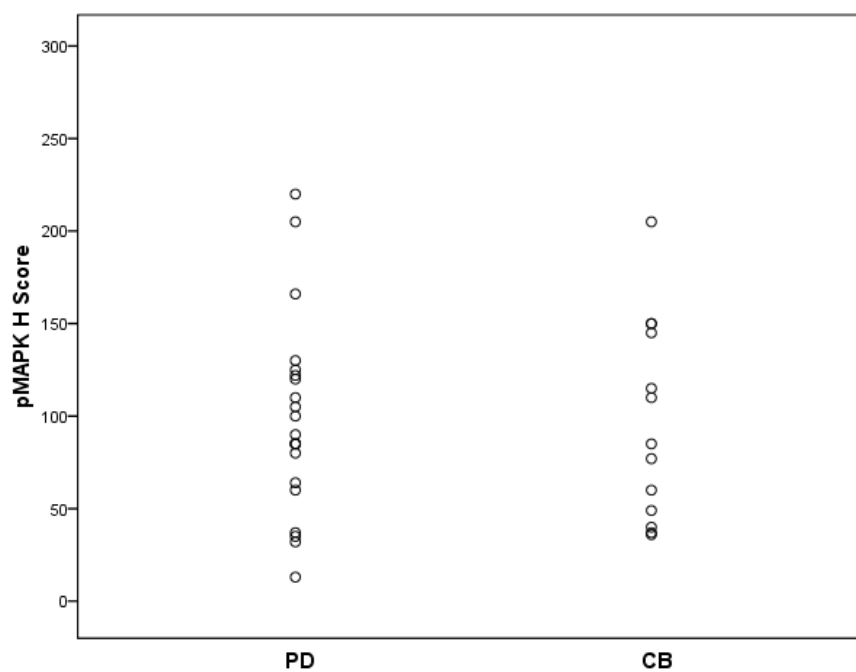


Figure 30: Pre-treatment (T0) pMAPK Expression by Response

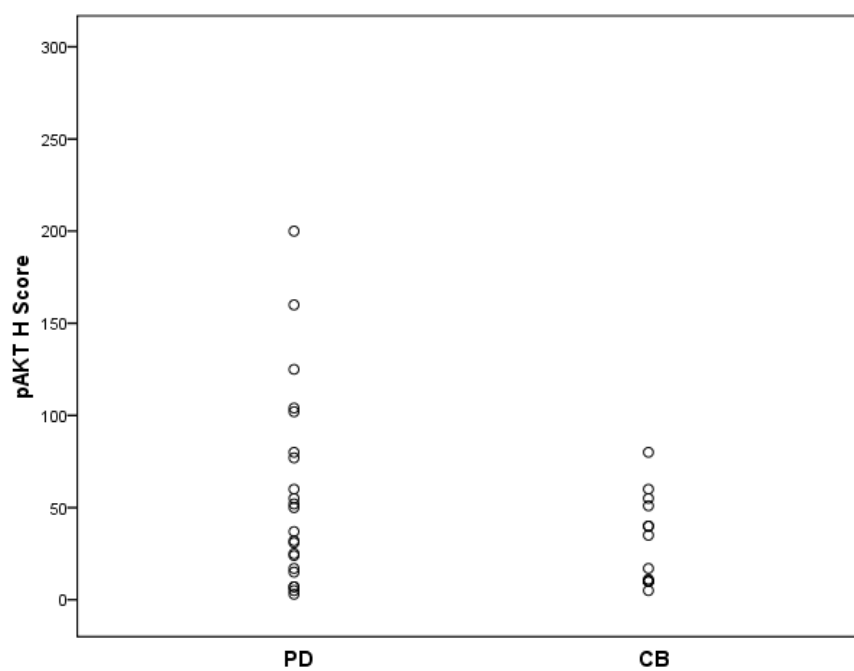


Figure 31: Pre-treatment (T0) pAKT Expression by Response

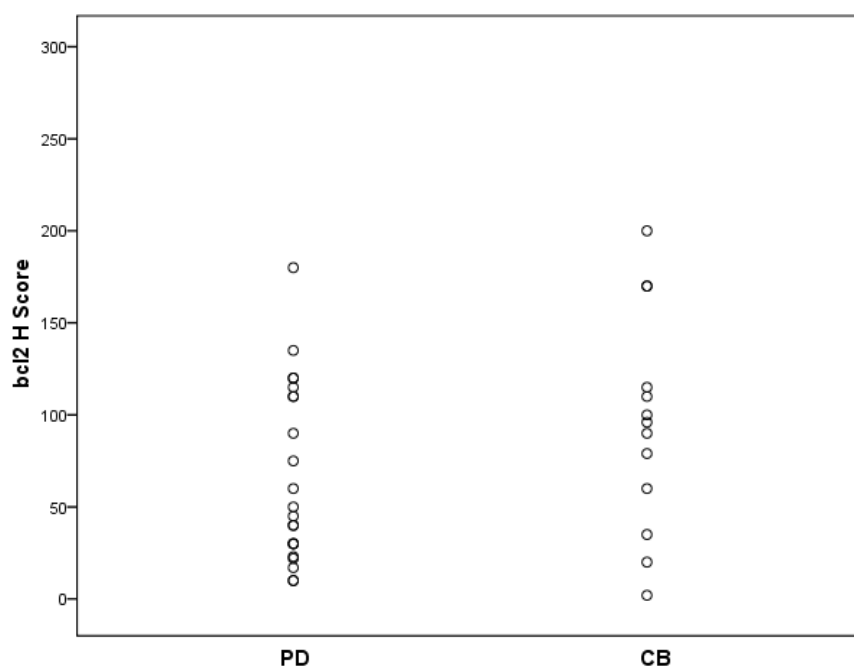


Figure 32: Pre-treatment (T0) bcl2 Expression by Response



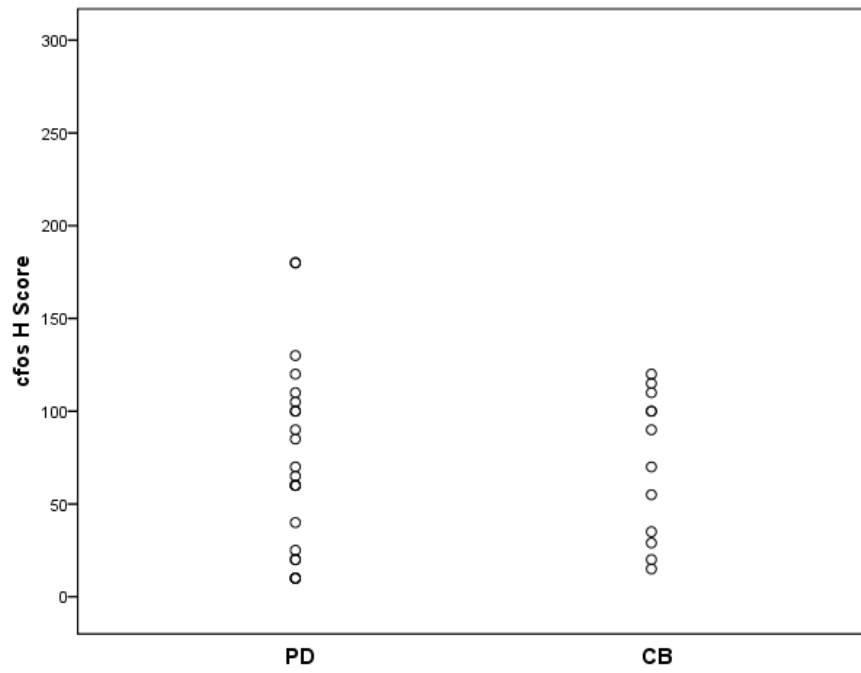


Figure 33: Pre-treatment (T0) cfos Expression by Response

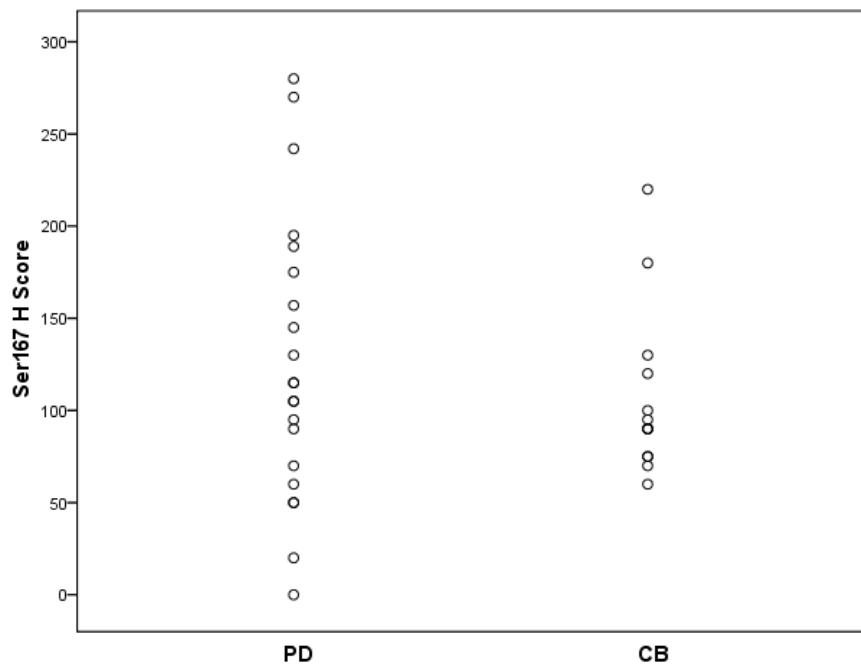


Figure 34: Pre-treatment (T0) pSer167 Expression by Response

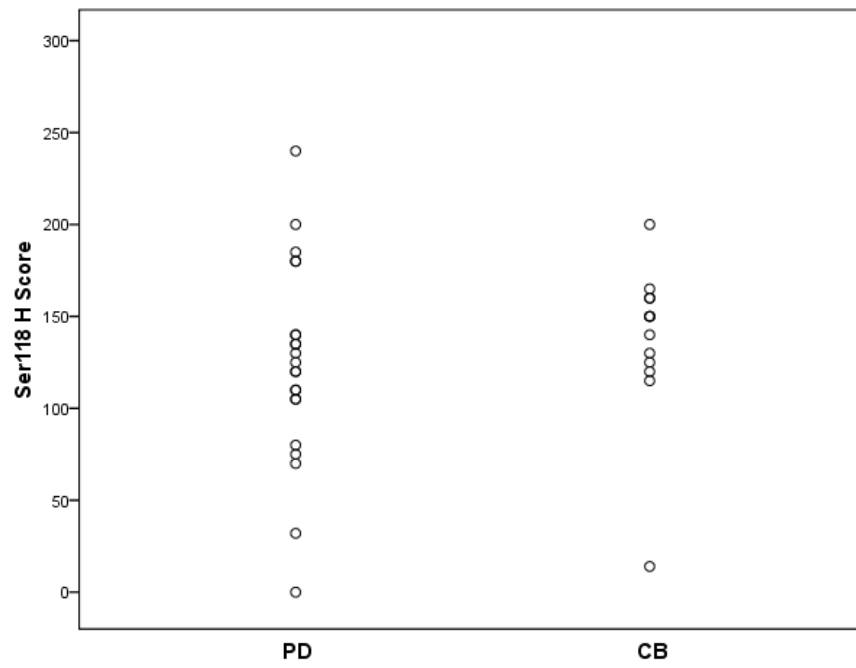


Figure 35: Pre-treatment (T0) pSer118 Expression by Response

## **10.7 Biomarker Changes on Treatment**

### **10.7.1 Pre-treatment to 8 weeks (T0 – T1)**

There was little gefitinib effect on the expression of ER (ER+ mean change -4.06, ER- mean change -1.25, mean difference -2.81,  $p > 1$  (Figure 36) and PgR (ER+ mean change -9.37, ER- mean change 0, mean difference -9.37,  $p > 1$  (Figure 37) in the two subgroups. EGFR increases were more often seen in the ER negative population which had initially expressed higher levels (ER+ mean change +1.78, ER- mean change +24.33,  $p = 0.75$  (Figure 38)) however pEGFR levels fell further in this subgroup (ER+ mean change -0.8, ER- mean change -10,  $p > 1$  (Figure 39). HER2 expression was reduced across the board (ER+ mean change -15.88, ER- mean change -18.33,  $p > 1$  (Figure 40)). pHER2 however was increased particularly in the ER negative samples (ER+ mean change +3.75, ER- mean change +26.67,  $p > 1$  (Figure 41)). Ki67 levels fell in the ER positive subjects whilst there was a small increase in the ER negative group (ER+ mean change -16, ER- mean change +2,  $p = 0.6$  (Figure 42)). IGFR (Figure 43), pIGFR (Figure 44), pMAPK (Figure 45) and pAKT (Figure 46) showed a small fall in mean expression in the ER positive samples. There was a greater fall in pMAPK (ER+ mean change -11.11, ER- mean change -18.5,  $p > 1$ ) and pAKT (ER+ mean change -10.25, ER- mean change -54.5,  $p = 0.75$ ) levels in the ER negative group but a small increase in IGFR and pIGFR. There were no striking differences in bcl2, cfos or Ser118 (Figures 47-49). In the ER negative patients we observed a large mean increase in pSer167 expression (ER+ mean change -8.57, ER- mean change +132,  $p = 0.24$  (Figure 50, Table 10)).

<b>Marker</b>	<b>ER positive change T0 - T1 (mean)</b>	<b>ER negative change T0 - T1 (mean)</b>	<b>Mean Difference</b>	<b>95% Confidence Interval for Difference</b>	<b>Mann Whitney on Changes T0 - T1 p value</b>	<b>Bonferroni Adjustment</b>
ER	-4.06	-1.25	-2.81	22.17 -- 27.73	0.74	>1
PgR	-9.37	0.00	-9.37	6.91 -- 25.68	0.23	>1
EGFR	1.78	24.33	-22.56	-0.11 -- 45.00	0.05	0.75
pEGFR	-0.80	-10.00	9.20	51.26 -- 32.86	0.58	>1
HER2	-15.88	-18.33	2.46	37.64 -- 32.73	0.85	>1
pHER2	3.75	26.67	-22.92	27.71 -- 73.50	0.30	>1
Ki67	-16.00	2.00	-18.00	0.21 -- 36.21	0.04	0.60
IGFR	-10.88	1.67	-12.54	26.87 -- 51.96	0.75	>1
pIGFR	-4.44	6.5	-10.94	19.74 -- 41.63	0.29	>1
pMAPK	-11.11	-18.5	7.39	98.22 -- 83.44	0.91	>1
pAKT	-10.25	-54.50	44.25	-77.72 - 10.78	0.05	0.75
Bcl2	-2.29	13.75	-16.04	18.85 -- 50.92	0.53	>1
cfos	8.71	9.25	-0.54	44.59 -- 45.62	0.93	>1
pSer167	-8.57	132	-140.57	-41.74 -- 239.40	0.016	0.24
pSer118	-16.67	19.5	-36.17	4.92 -- 77.28	0.09	>1

Table 10: Mean Changes from Pre-treatment (T0) to 8 weeks (T1)

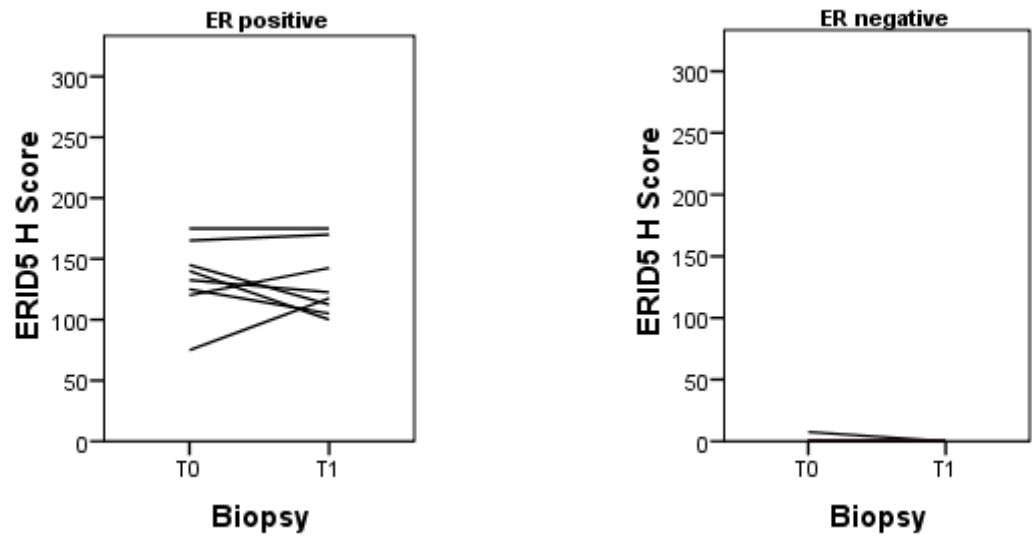


Figure 36: Changes in ER Expression from Pre-treatment (T0) to 8 weeks (T1)

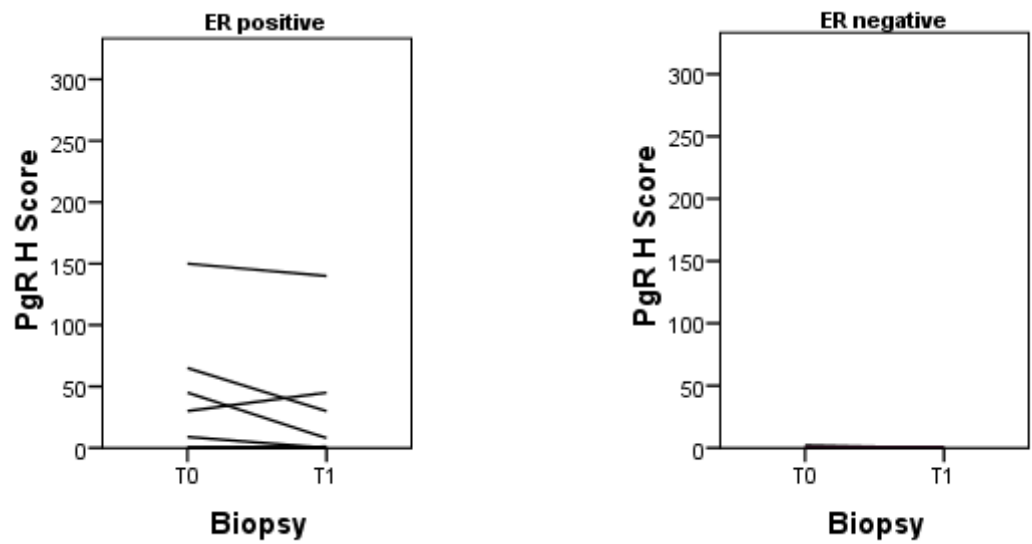


Figure 37: Changes in PgR Expression from Pre-treatment (T0) to 8 weeks (T1)

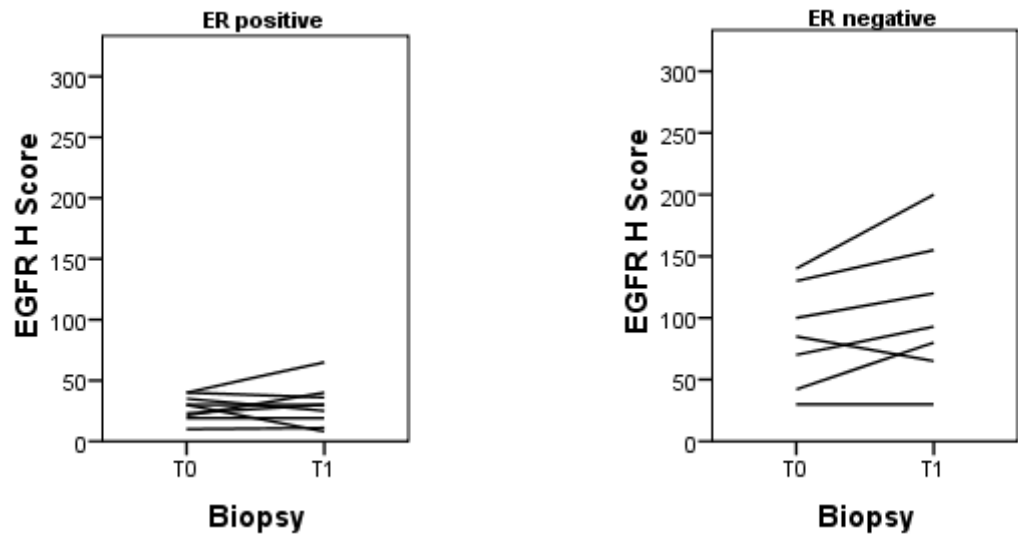


Figure 38: Changes in EGFR Expression from Pre-treatment (T0) to 8 weeks (T1)

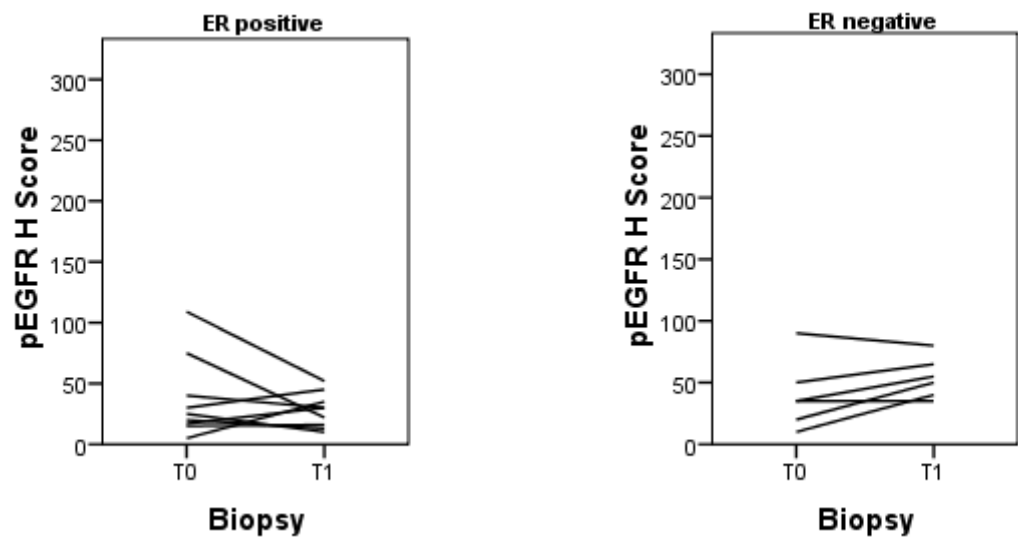


Figure 39: Changes in pEGFR Expression from Pre-treatment (T0) to 8 weeks (T1)

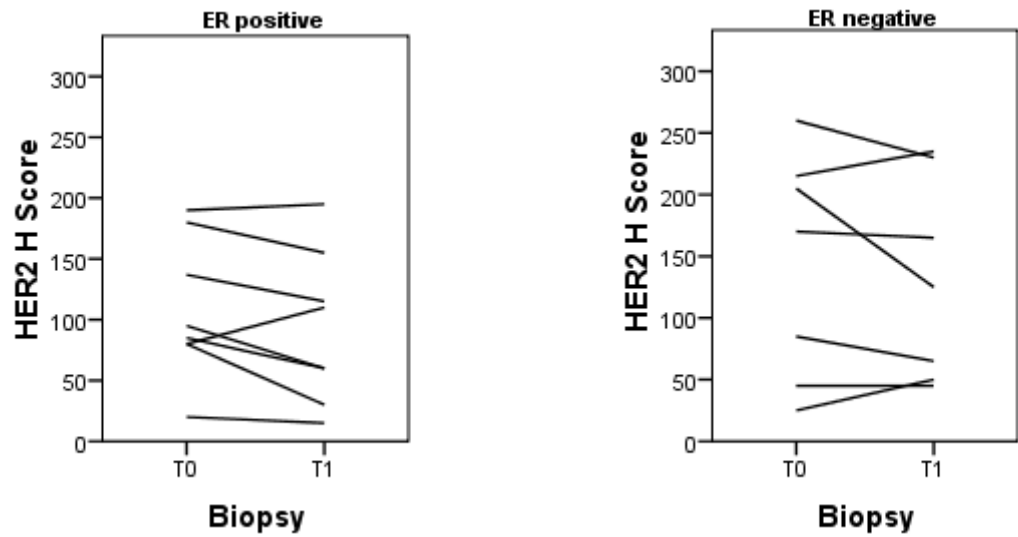


Figure 40: Changes in HER2 Expression from Pre-treatment (T0) to 8 weeks (T1)

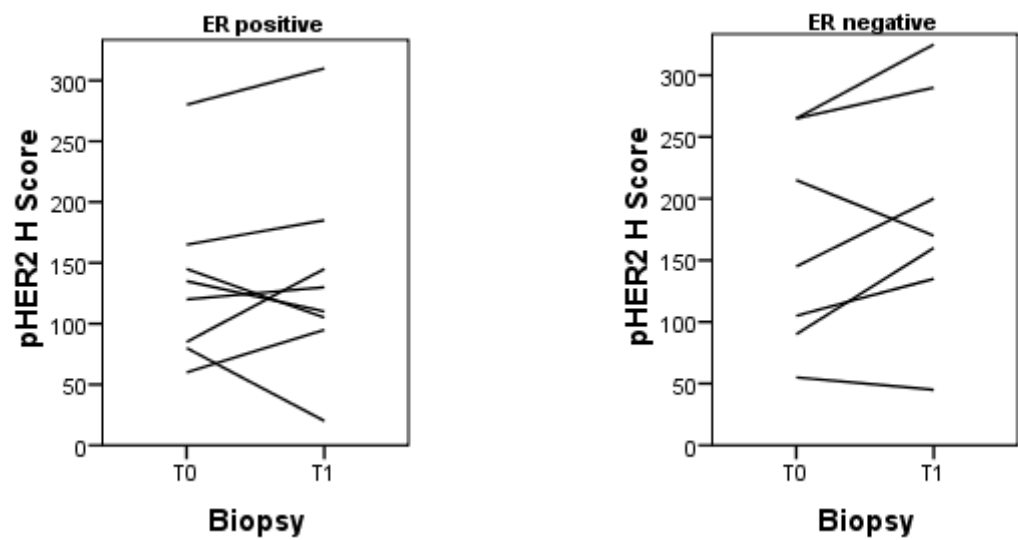


Figure 41: Changes in pHER2 Expression from Pre-treatment (T0) to 8 weeks (T1)

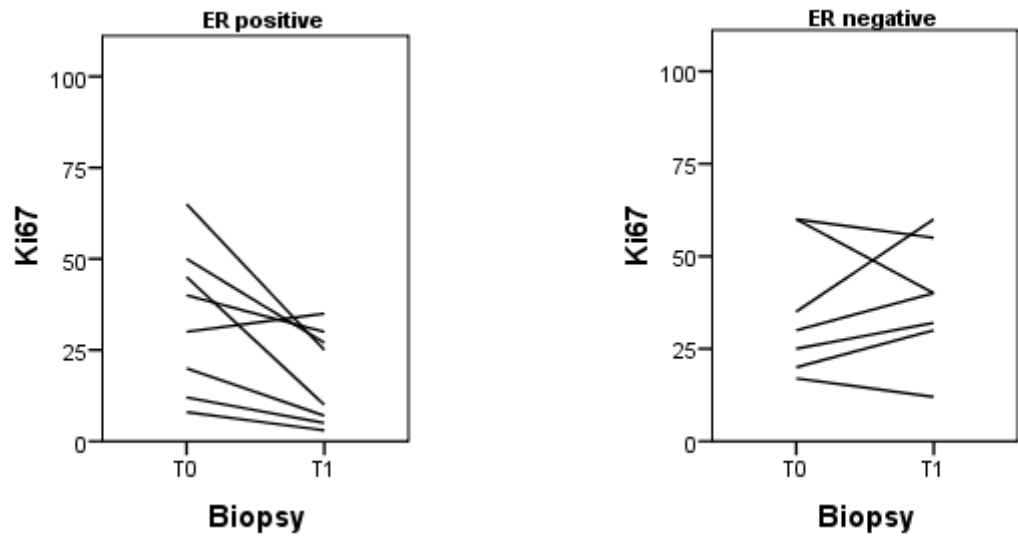


Figure 42: Changes in Ki67 Expression from Pre-treatment (T0) to 8 weeks (T1)

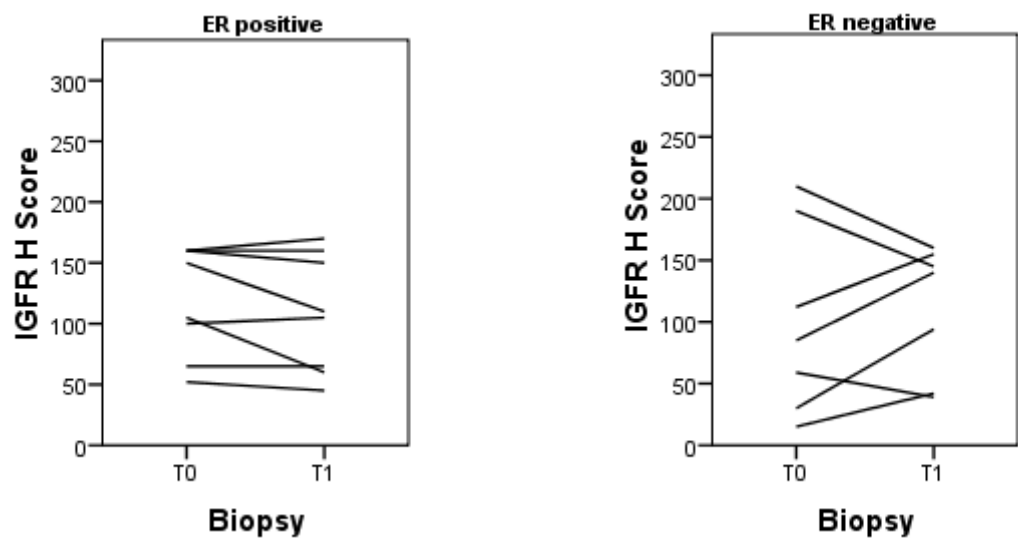


Figure 43: Changes in IGFR Expression from Pre-treatment (T0) to 8 weeks (T1)



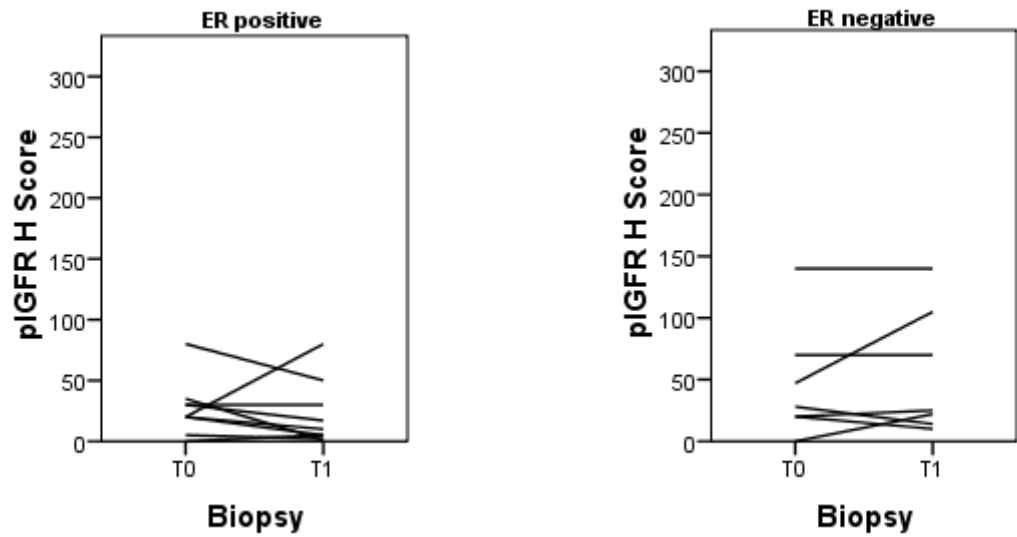


Figure 44: Changes in pIGFR Expression from Pre-treatment (T0) to 8 weeks (T1)

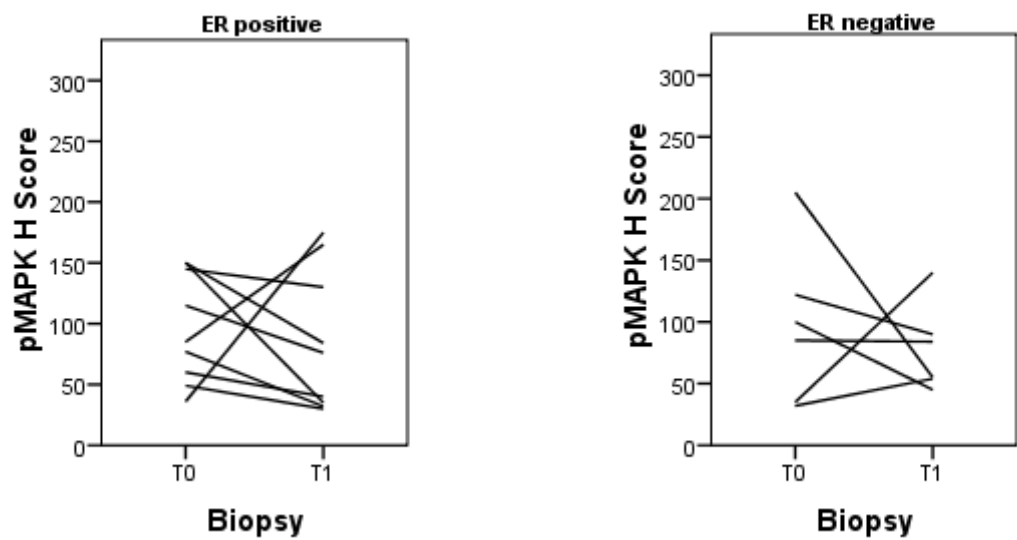


Figure 45: Changes in pMAPK Expression from Pre-treatment (T0) to 8 weeks (T1)

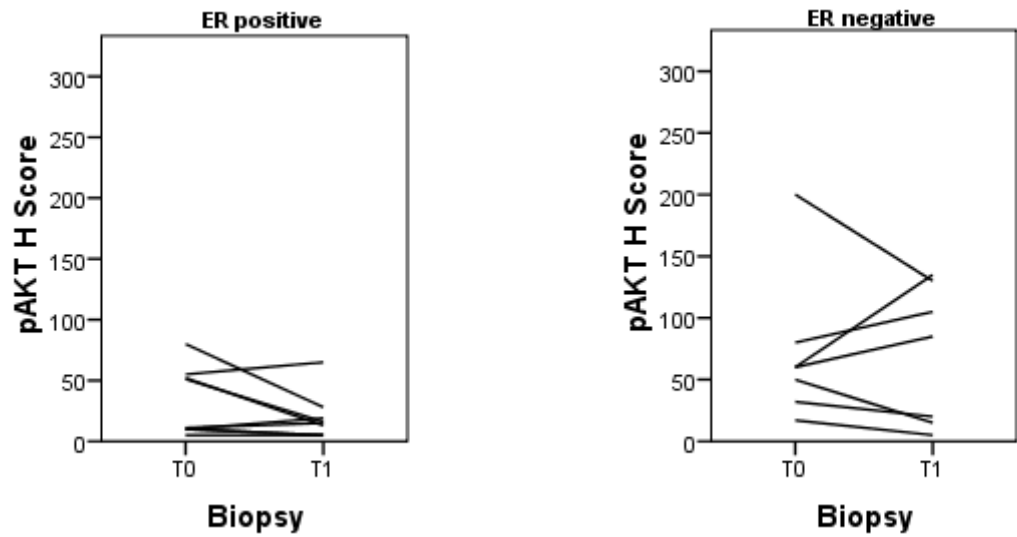


Figure 46: Changes in pAKT Expression from Pre-treatment (T0) to 8 weeks (T1)

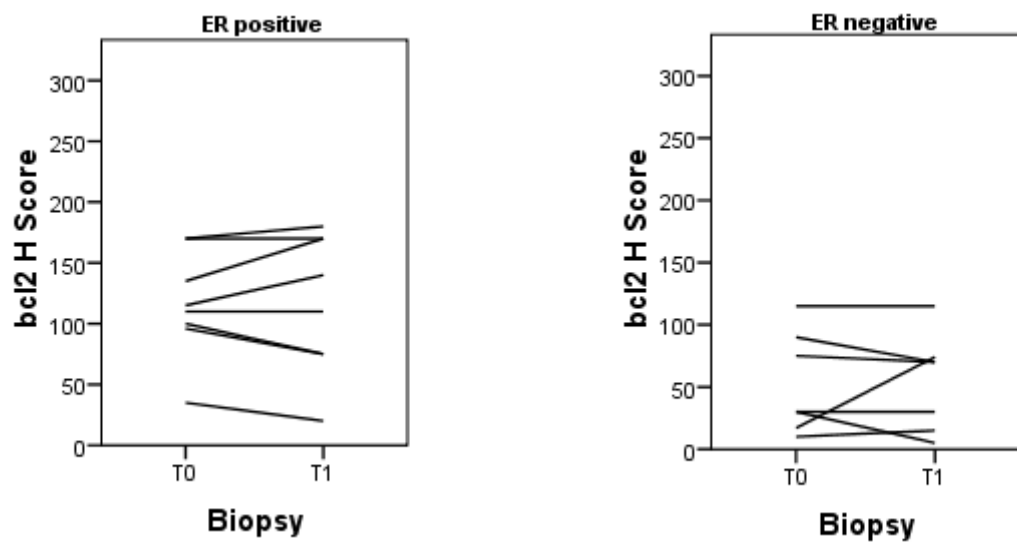


Figure 47: Changes in bcl2 Expression from Pre-treatment (T0) to 8 weeks (T1)

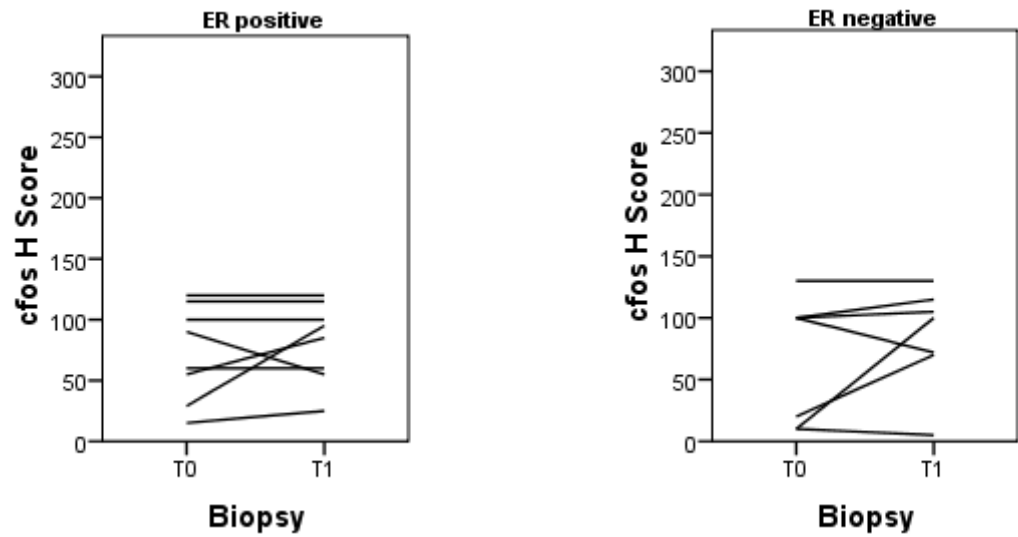


Figure 48: Changes in c-fos Expression from Pre-treatment (T0) to 8 weeks (T1)

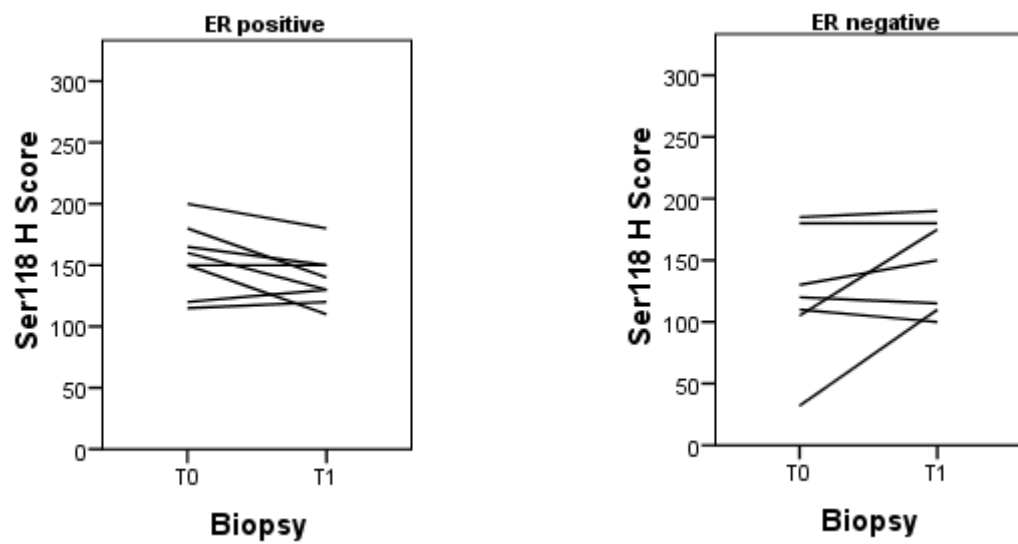


Figure 49: Changes in pSer118 Expression from Pre-treatment (T0) to 8 weeks (T1)

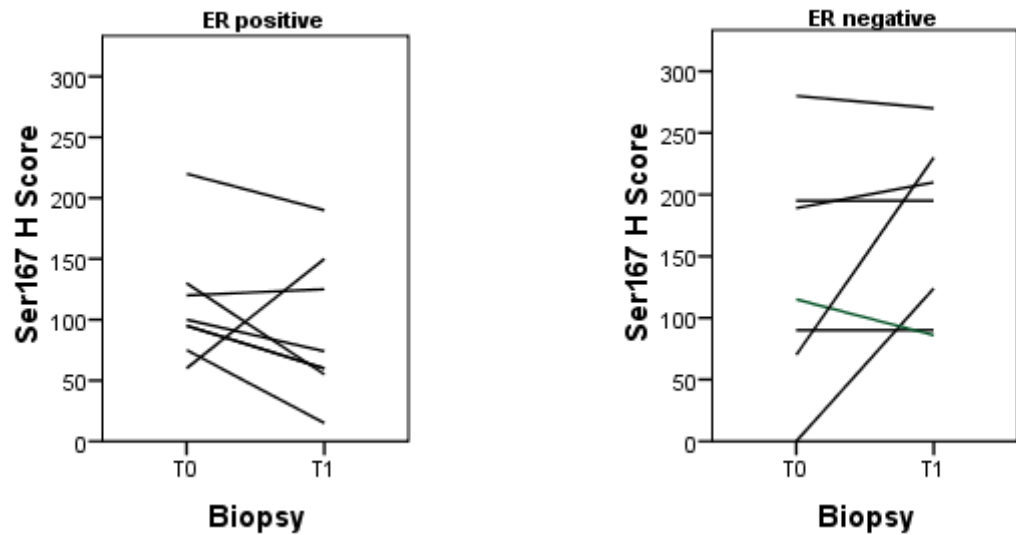


Figure 50: Changes in pSer167 Expression from Pre-treatment (T0) to 8 weeks (T1)

### 10.7.1.1 For Those Achieving Clinical Benefit

There was a T0-T1 decline in mean Ki67 levels (33.17 – 18.38) with CB but no significant fall at T1 with PD (38.85 – 37.71) ( $p=0.024$ ) (Figure 51).

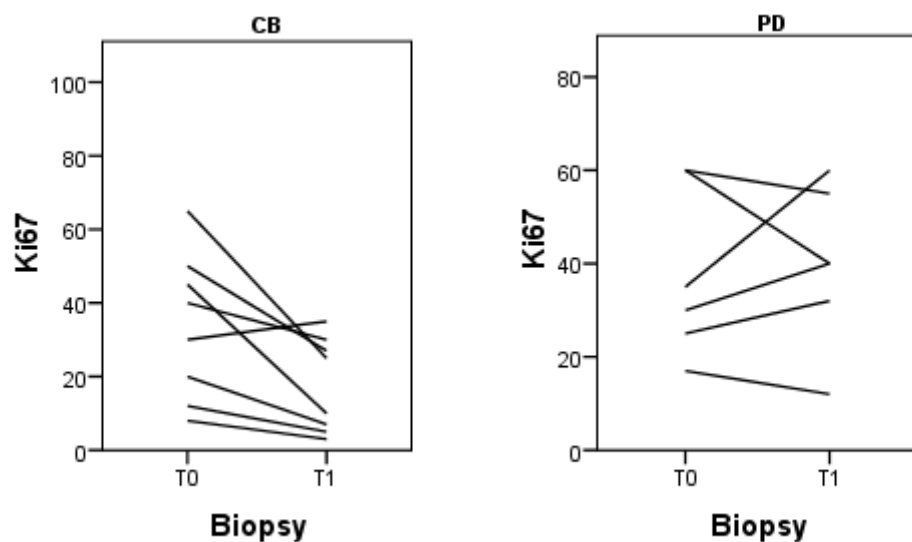


Figure 51: Changes in Ki67 Expression from Pre-treatment (T0) to 8 weeks (T1) by Response Group

Declines in Ki67 positivity of >10% were common in patients achieving CB, but were rarely observed in patients with PD. The mean T0-T1 change in Ki67 was -59.9% and +9.8% in patients with CB and PD, respectively. At T1 the responders exhibited significantly lower Ki67 expression than the early progressors (p=0.019).

Matched analysis revealed that five of the patients achieving CB showed a >10% T0-T1 fall in pEGFR and further biomarker examination in these patients revealed decreases in phosphorylation of the downstream signalling element MAPK and Ki67 proliferative capacity also occurred (Figure 52 a - c) (Table 11). There were no obvious differences or trends in expression of activation of any of the other markers.

Pt no.	Activated EGFR		Activated MAPK		Ki67		T0-T1 change observed
	T0	T1	T0	T1	T0	T1	
1	40	30	150	84	50	27	All decrease
2	20	13	115	76	40	30	All decrease
3	25	10	150	35	12	5	All decrease
4	75	22	77	32	65	25	All decrease
5	109	52	36	175	45	10	All decrease (except Activated MAPK)

Table 11: Changes in pEGFR, pMAPK and Ki67 in 5 CB patients

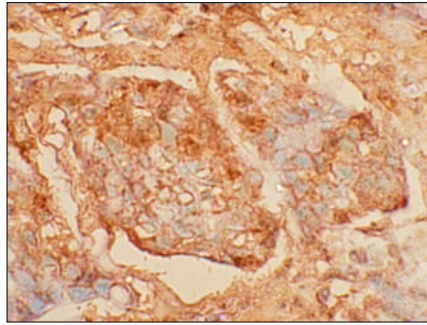


Fig a Activated EGFR at T0

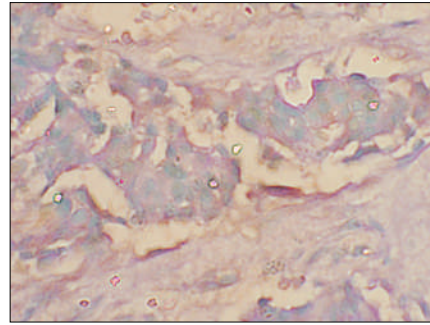


Fig b Activated EGFR at T1

Figure 52a: Changes in pEGFR Staining Pre-treatment (T0) – 8 weeks (T1)

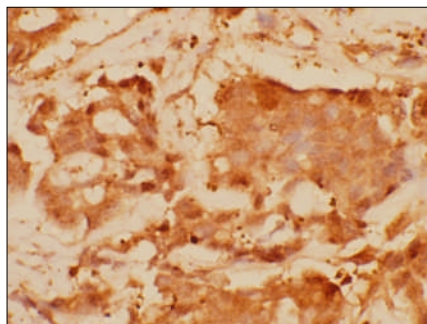


Fig a Activated MAPK at T0

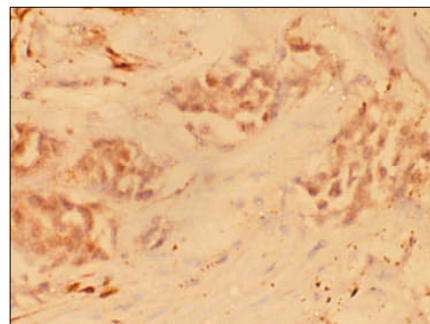


Fig b Activated MAPK at T1

Figure 52b: Changes in pMAPK Staining Pre-treatment (T0) – 8 weeks (T1)

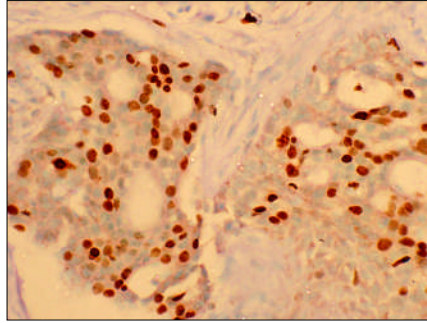


Fig a Ki67 at T0

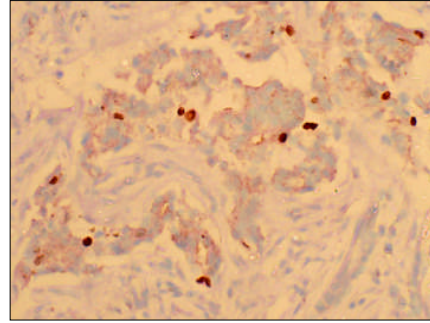


Fig b Ki67 at T1

Figure 52c: Changes in Ki67 Staining Pre-treatment (T0) – 8 weeks (T1)

### **10.7.1.2 For Those with Early Progression**

We observed an increase in total EGFR expression at T1 in the PD subset only (T0 94, 42-100. T1 119, 65-200). We also observed an increase in pHER2 levels (T0 60, 20-115 T1 74, 20-125). Given the general increase in these type 1 markers it seems likely that the lack of statistical significance is due to the small number of patients and biopsies. There were no striking changes in expression or activation of Ser167, Ser116, MAPK, AKT, bcl2, c-fos, IGFR or Ki67.

### **10.7.2 Pre-treatment to 6 months (T0 -T2)**

All patients with biopsies at 6 months were responders and all but one was ER positive. In this ER positive subgroup ER expression was unaffected (Figure 53), PgR expression was increased with a mean change from pre-treatment of +32.67 (Figure 54). EGFR expression increased by a mean of +11.83 (Figure 55) whilst pEGFR expression was unchanged (Figure 56). We observed a mean change from baseline of -37 to decrease HER2 expression (Figure 57) but conversely a mean change of +22.5 to increase its activated form (Figure 58). The mean change of -9.17 in Ki67 levels lead to a small decrease from baseline (Figure 59). The downstream effectors all had mean increases (IGFR +30.83, pIGFR +60.5, pMAPK +32.8, pAKT 26.7 (Figures 60 - 63). bcl2 expression was somewhat increased in both groups (Figure 64). cfos levels had a mean increase from baseline in the ER positive group (Figure 65). pSer118 and pSer167 had minor increases in their mean expression (Figures 66 – 67). It is difficult to comment on the changes seen in the single ER negative patient as they may not be representative of the changes seen if we had had a larger



sample group. Hence these are simply shown in the table and graphically (Table 12).

Marker	ER positive change T0 - T2 (mean)	ER negative change T0 - T2 (mean)	Mean Difference between ER positive and ER negative change T0 - T2	95% Confidence Interval for Difference
ER	0.83	0.00	0.83	127.29 – -125.64
PgR	32.67	0.00	32.67	247.92 – -182.53
EGFR	11.83	75.00	63.17	43.51 – -169.83
pEGFR	1.00	80	79	-20.20 – 120.86
HER2	-37.00	35.00	-72.00	35.96 – -179.92
pHER2	22.50	67.00	-44.5	89.50 – -178.42
Ki67	-9.17	-40.00	30.83	114.32 – -52.68
IGFR	30.83	10.00	-20.83	123.76 – -82.09
pIGFR	60.50	105.00	-44.5	190.82 – -279.81
pMAPK	32.80	-75.00	-107.83	200.31 - 15.35
pAKT	26.70	10	16.7	-50.03 – 25.45
Bcl2	13.50	13.00	0.50	150.57 – -149.57
cfos	26.25	0.00	26.24	121.92 – -69.47
pSer167	18.75	31.00	-12.25	222.59 – -247.10
pSer118	21.25	30.00	-8.75	123.09 – -140.56

Table 12: Mean Changes from Pre-treatment (T0) to 6 months (T2)

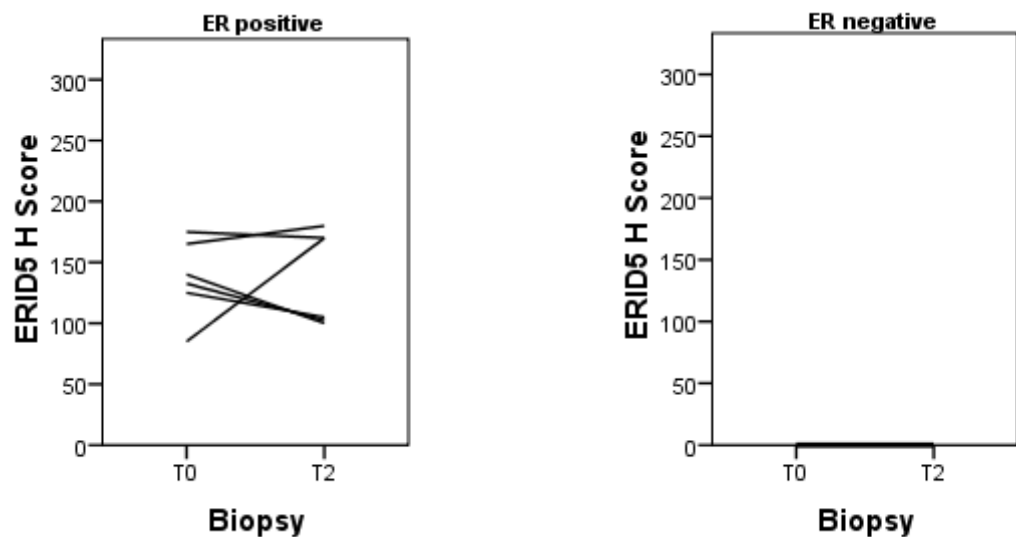


Figure 53: Changes in ER Expression from Pre-treatment (T0) to 6 months (T2)

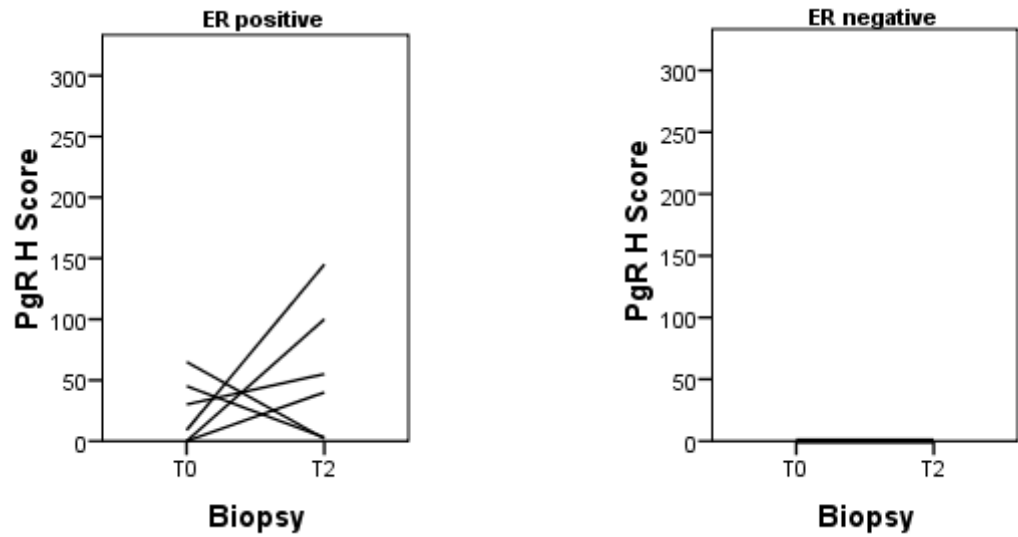


Figure 54: Changes in PgR Expression from Pre-treatment (T0) to 6 months (T2)

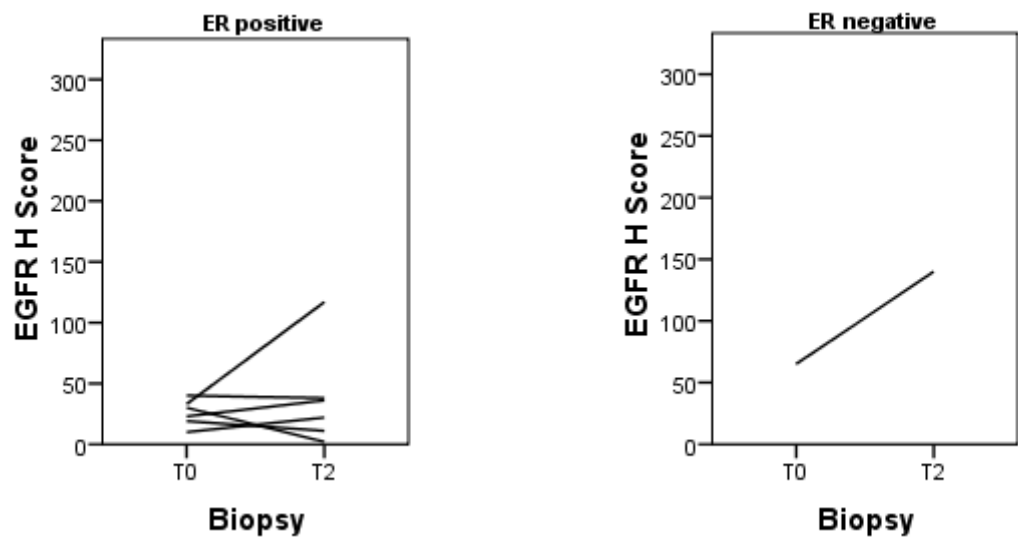


Figure 55: Changes in EGFR Expression from Pre-treatment (T0) to 6 months (T2)

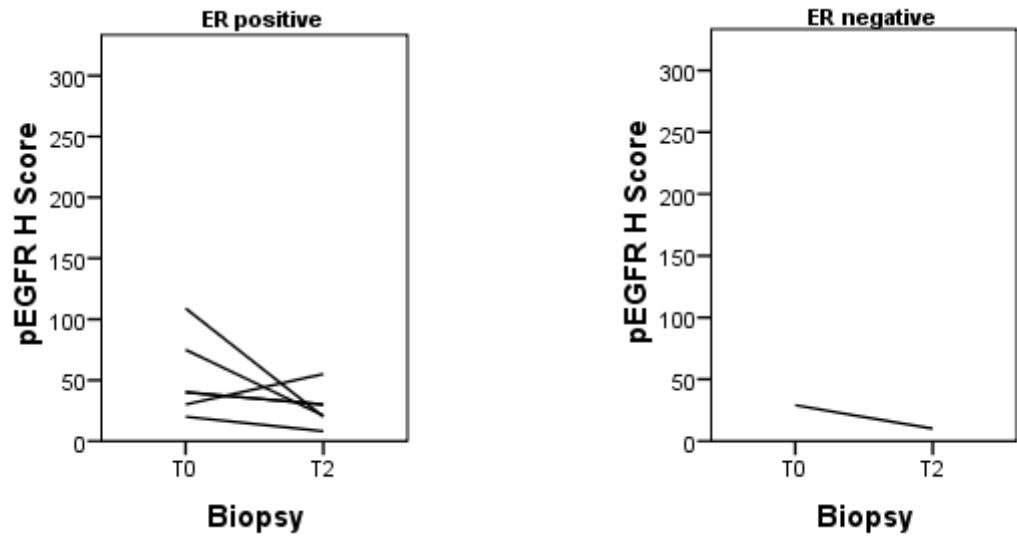


Figure 56: Changes in pEGFR Expression from Pre-treatment (T0) to 6 months (T2)

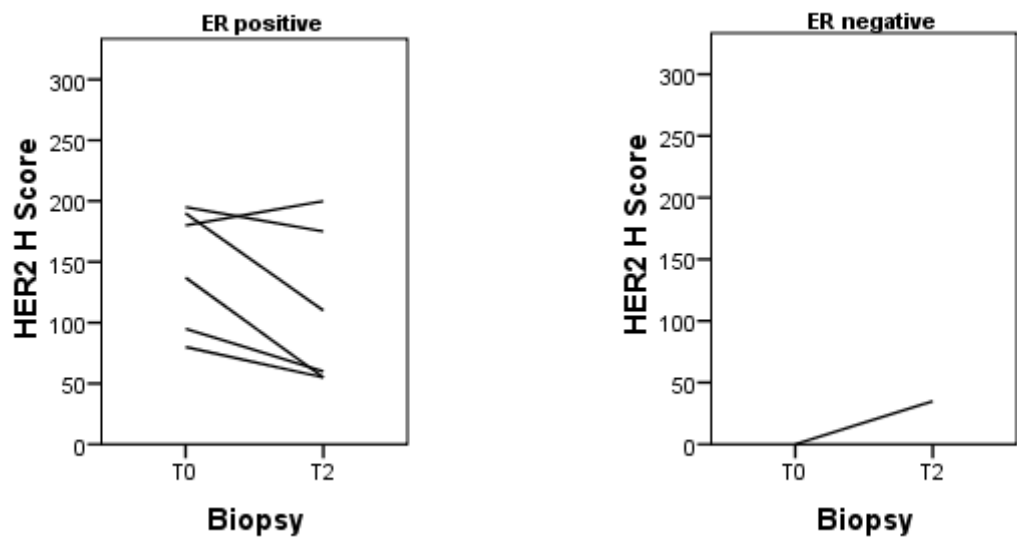


Figure 57: Changes in HER2 Expression from Pre-treatment (T0) to 6 months (T2)

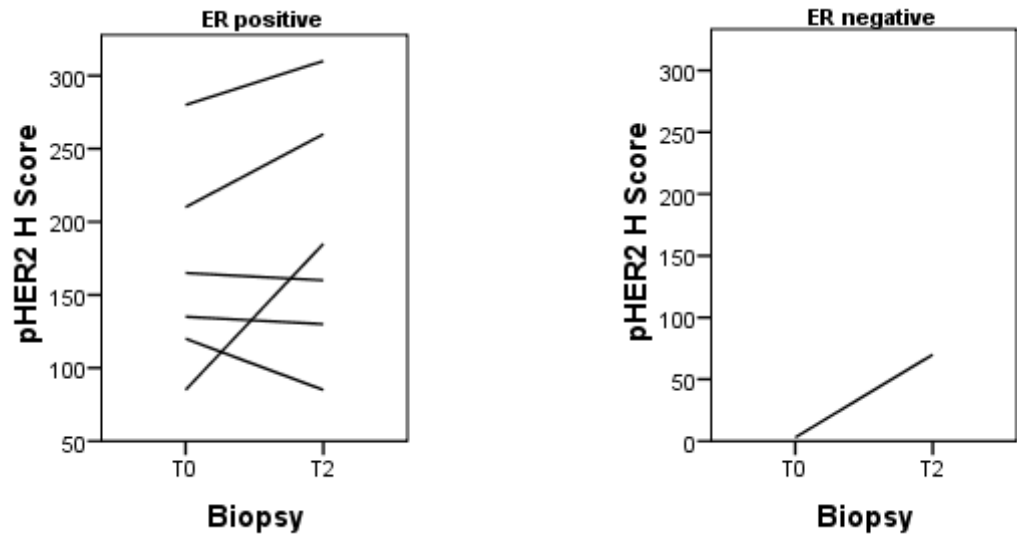


Figure 58: Changes in pHER2 Expression from Pre-treatment (T0) to 6 months (T2)

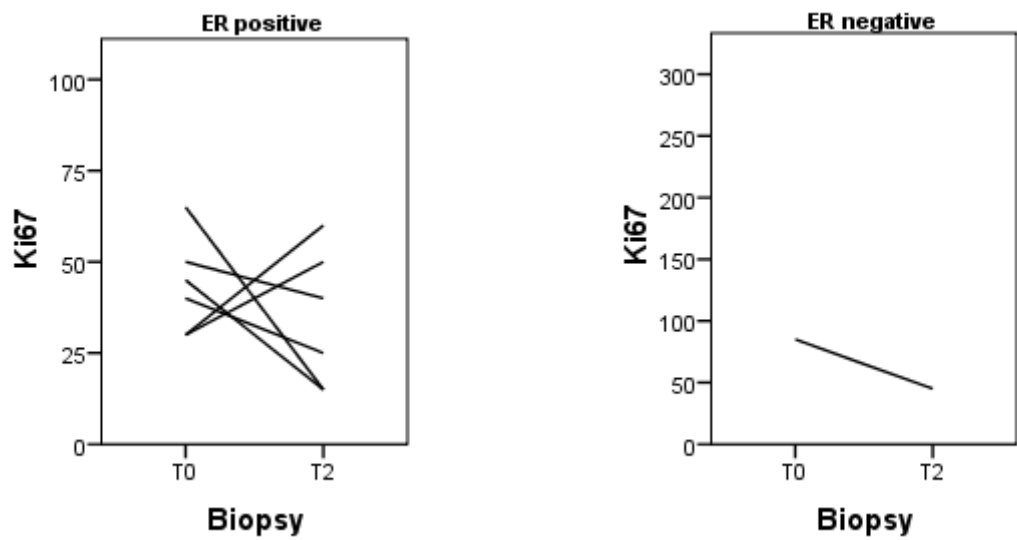


Figure 59: Changes in Ki67 Expression from Pre-treatment (T0) to 6 months (T2)

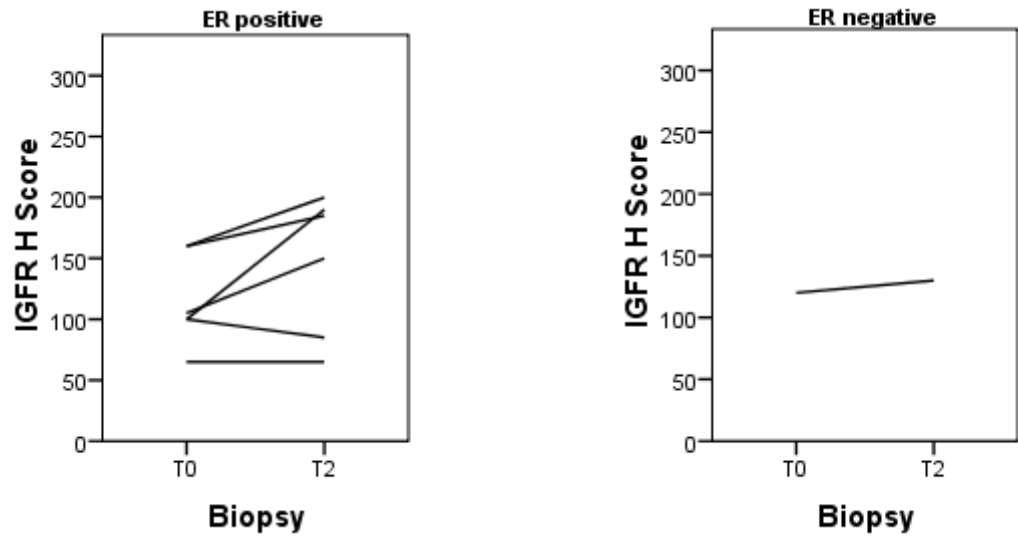


Figure 60: Changes in IGFR Expression from Pre-treatment (T0) to 6 months (T2)

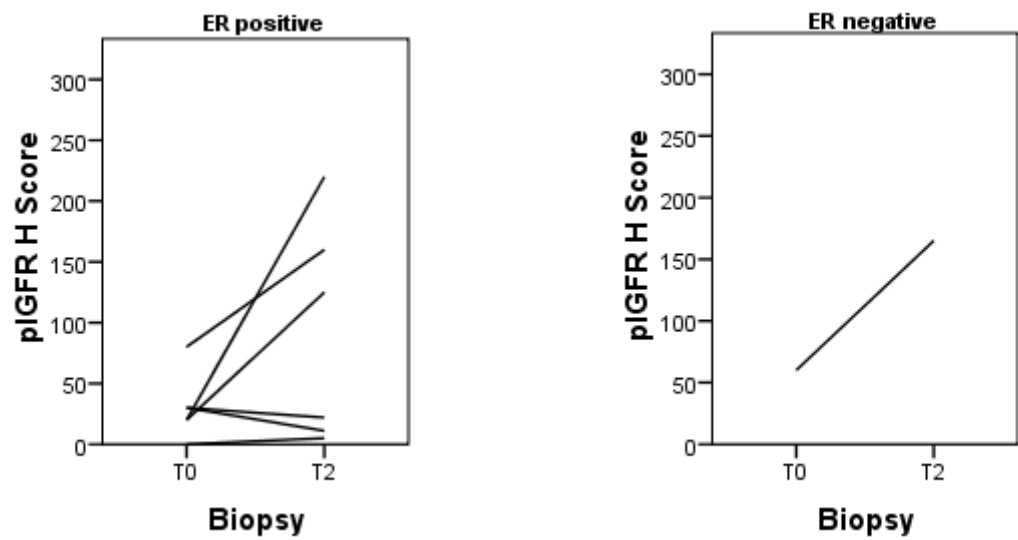


Figure 61: Changes in pIGFR Expression from Pre-treatment (T0) to 6 months (T2)

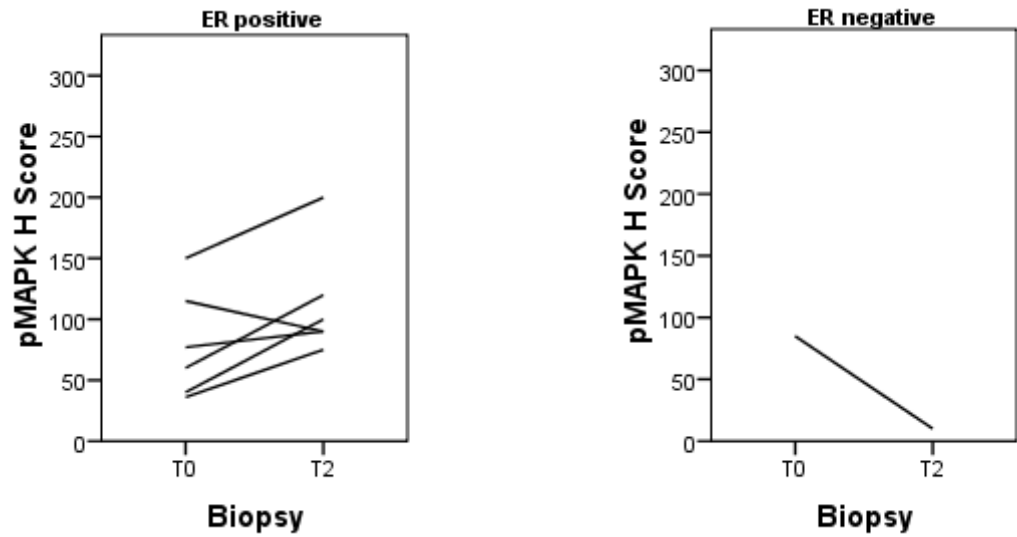


Figure 62: Changes in pMAPK Expression from Pre-treatment (T0) to 6 months (T2)

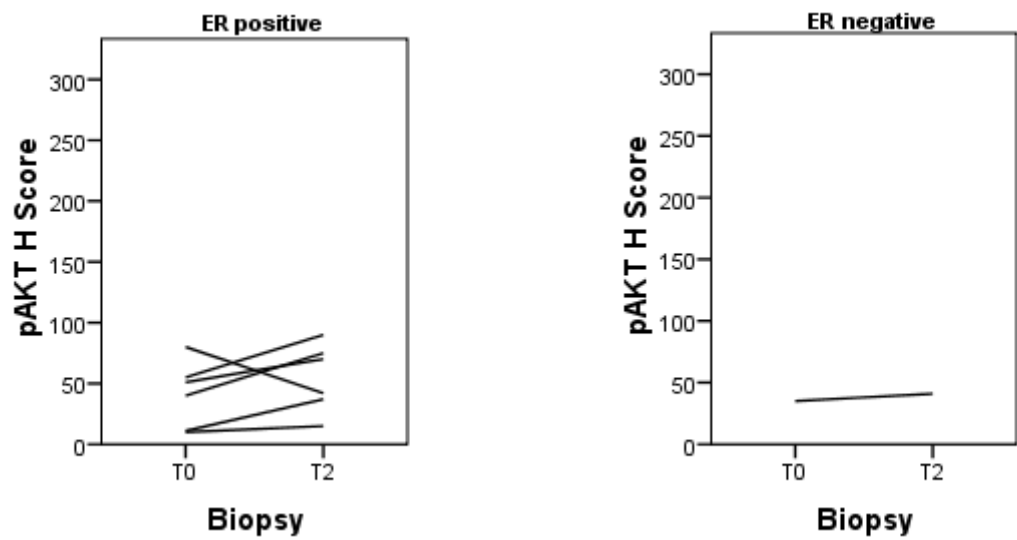


Figure 63: Changes in pAKT Expression from Pre-treatment (T0) to 6 months (T2)

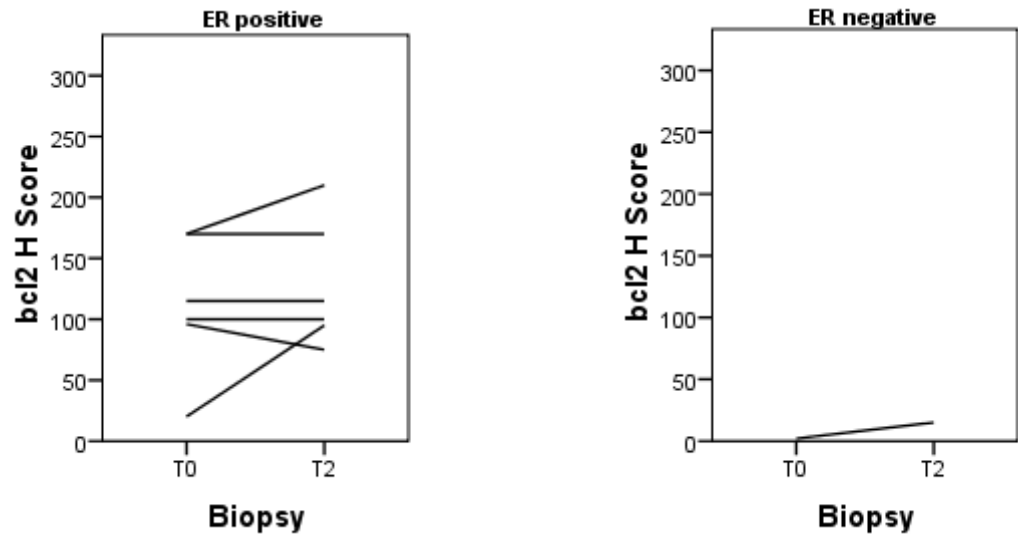


Figure 64: Changes in bcl2 Expression from Pre-treatment (T0) to 6 months (T2)

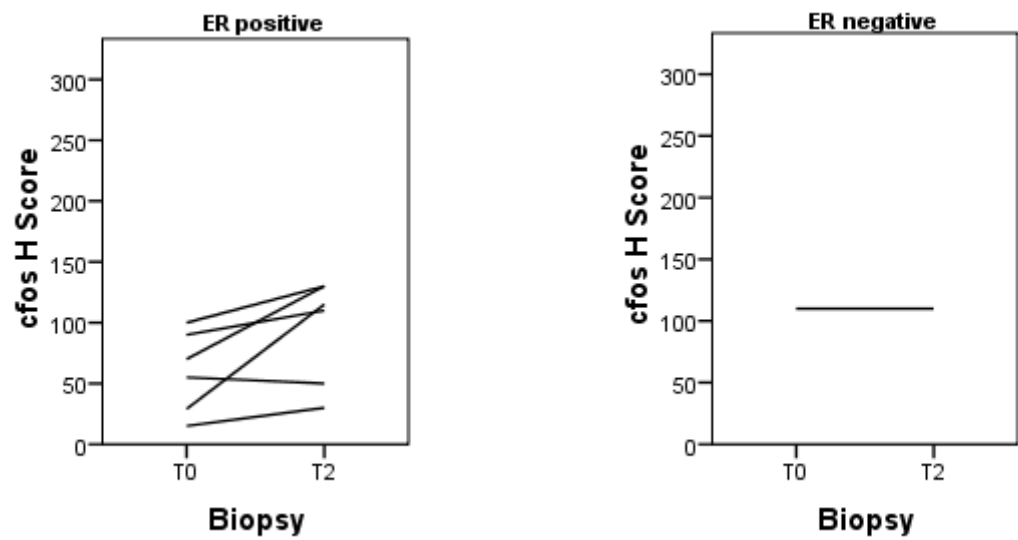


Figure 65: Changes in cfos Expression from Pre-treatment (T0) to 6 months (T2)

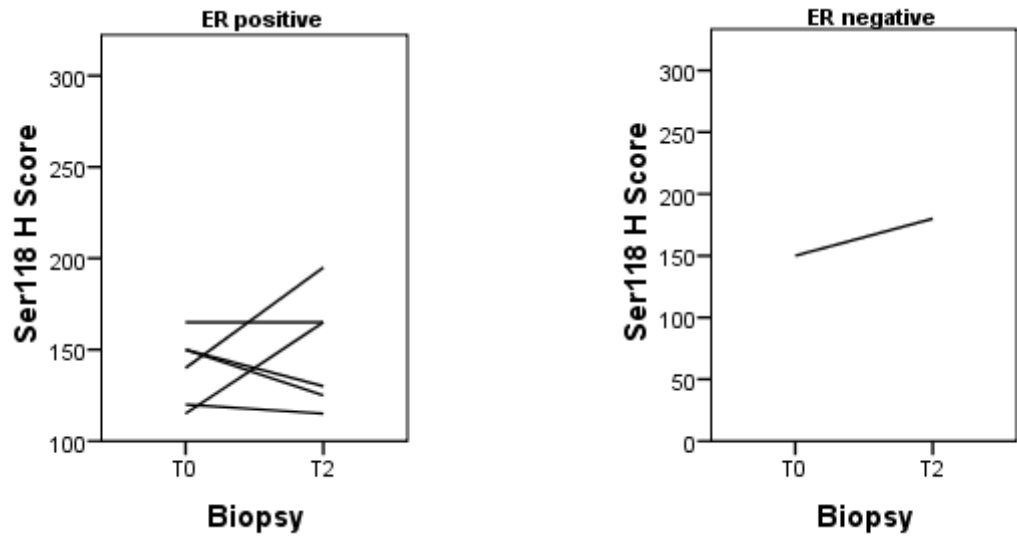


Figure 66: Changes in pSer118 Expression from Pre-treatment (T0) to 6 months (T2)

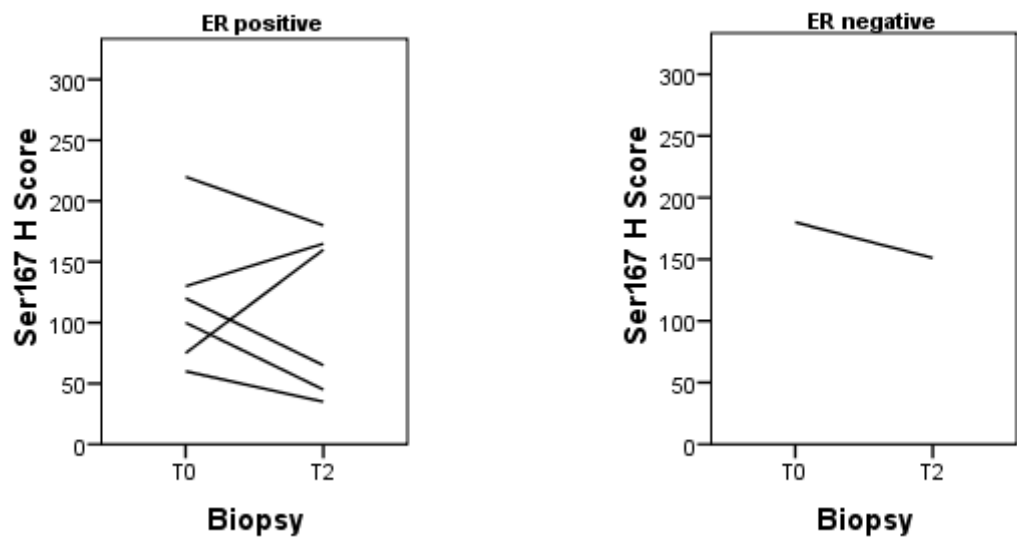


Figure 67: Changes in pSer167 Expression from Pre-treatment (T0) to 6 months (T2)



### 10.7.3 Pre-treatment to Progression (T0-T3)

At the development of resistance the ER positive samples a mean change from baseline to increase ER (Figure 68) and PgR expression (Figure 69). In the both groups the mean change in EGFR from baseline was an increase (Figure 70) whilst its activated form had a mean decrease (Figure 71). The mean change in Her2 expression was a decrease from baseline in the ER positive group but an increase in the ER negative group (Figure 72), whilst the mean change in pHER2 expression was an increase across the board (Figure 73). The ER positive samples showed a mean increase in Ki67 vs. pre-treatment whilst the ER negative samples were essentially unchanged (Figure 74). We observed mean changes in IGFR and pIGFR to increase expression particularly in the ER positive patients (Figure 75 – 76) who also revealed some increases in pMAPK and pAKT (Figure 77 – 78). bcl2 levels were unaffected (Figure 79). cfos expression had a mean increase in the ER positive patients and a fall in the ER negative group (Figure 80). pSer118 levels were decreased in both groups (Figure 81). The ER negative group had a mean change from baseline to decrease pMAPK with a large mean changes to increase pAKT and Ser167 expression (Figure 82) (Table 13).

Marker	ER positive change from baseline (mean)	ER negative change from baseline (mean)	Mean Difference between ER positive and ER negative change from baseline	95% Confidence Interval for Difference
ER	25.83	-0.86	26.69	79.95 – -26.64
PgR	69.67	0.00	69.67	128.62 - 10.75
EGFR	26.00	31.29	-5.29	54.74 – -65.32
pEGFR	0.00	-20	-20	-35 - 20
HER2	-26.67	30.00	-56.67	14.09 – -127.46
pHER2	25.00	17.71	7.29	78.45 – -63.89
Ki67	15.0	-3.28	18.29	52.52 – -15.91
IGFR	45.00	16.43	28.57	123.78 – -66.63
pIGFR	51.67	7.43	44.24	108.23 – -19.75
pMAPK	9.67	-30.00	39.67	123.96 – -44.63
pAKT	14.67	115.00	-100.33	103.84 – -304.54
Bcl2	2.50	-4.83	7.33	38.87 – -24.2
cfos	15.50	-15.00	30.5	96.82 – -35.80
pSer167	0.00	84.17	-84.17	5.07 – -173.41
pSer118	-17.50	-5.83	-11.67	54.76 – -78.10

Table 13: Mean Changes from Pre-treatment (T0) to Progression (T3)

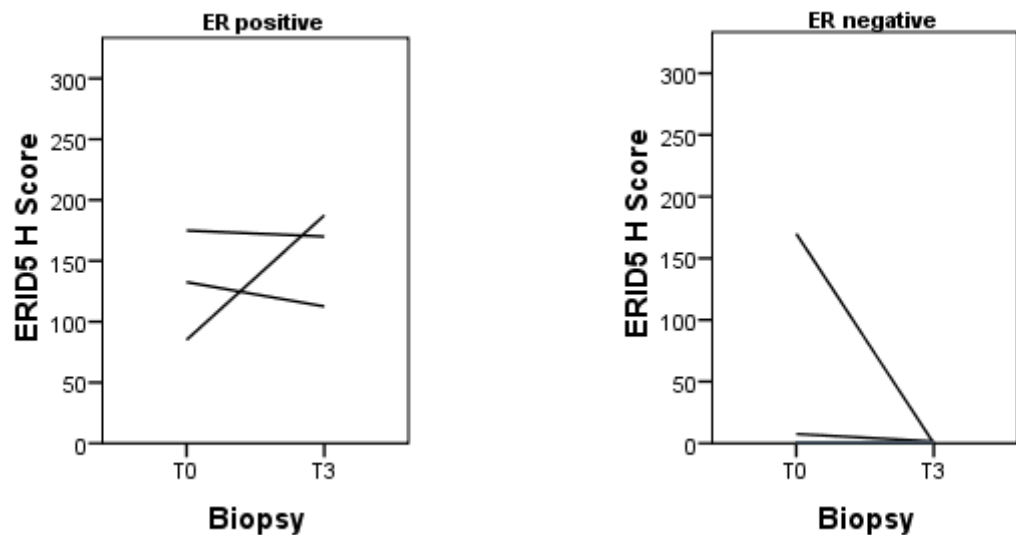


Figure 68: Changes in ER Expression from Pre-treatment (T0) to Progression (T3)

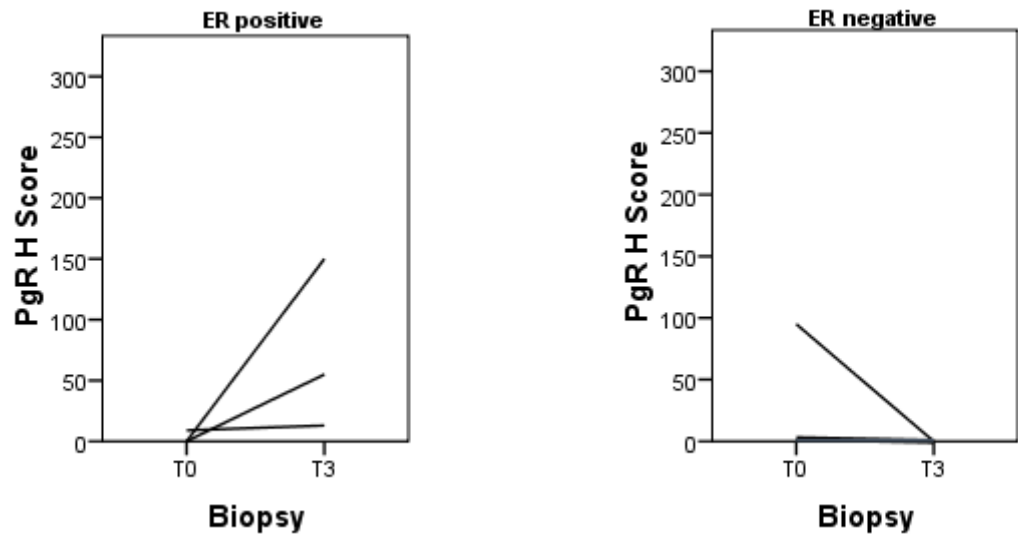


Figure 69: Changes in PgR Expression from Pre-treatment (T0) to Progression (T3)

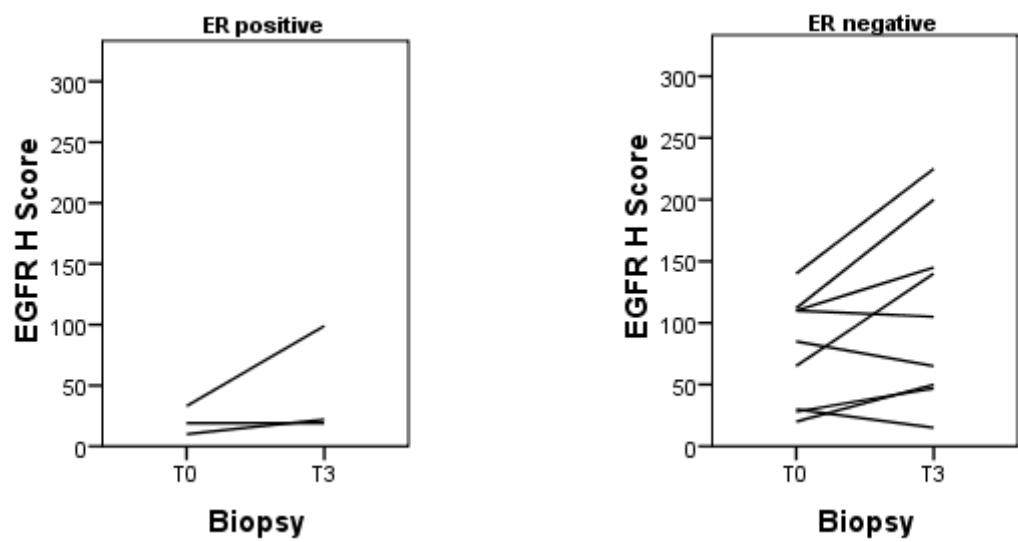


Figure 70: Changes in EGFR Expression from Pre-treatment (T0) to Progression (T3)

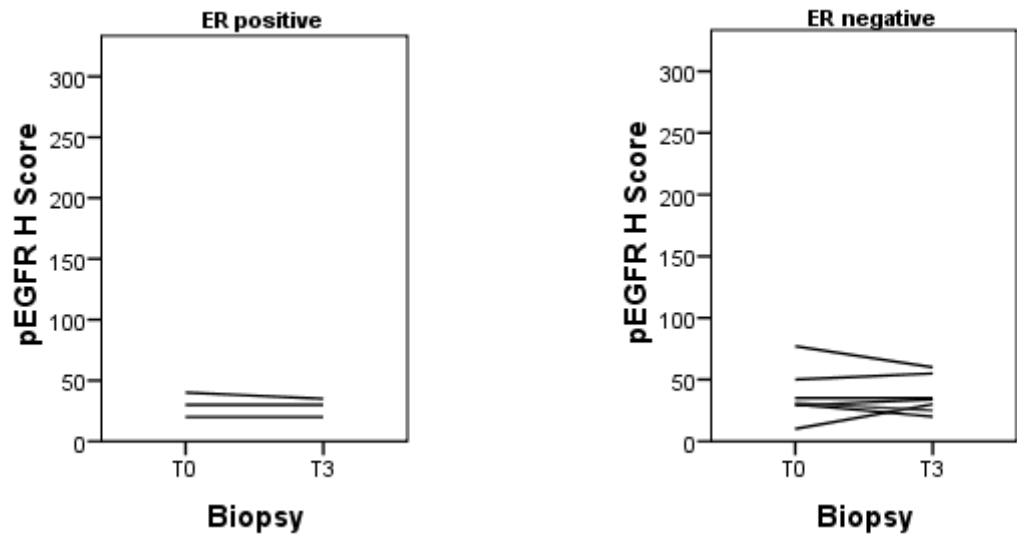


Figure 71: Changes in pEGFR Expression from Pre-treatment (T0) to Progression (T3)

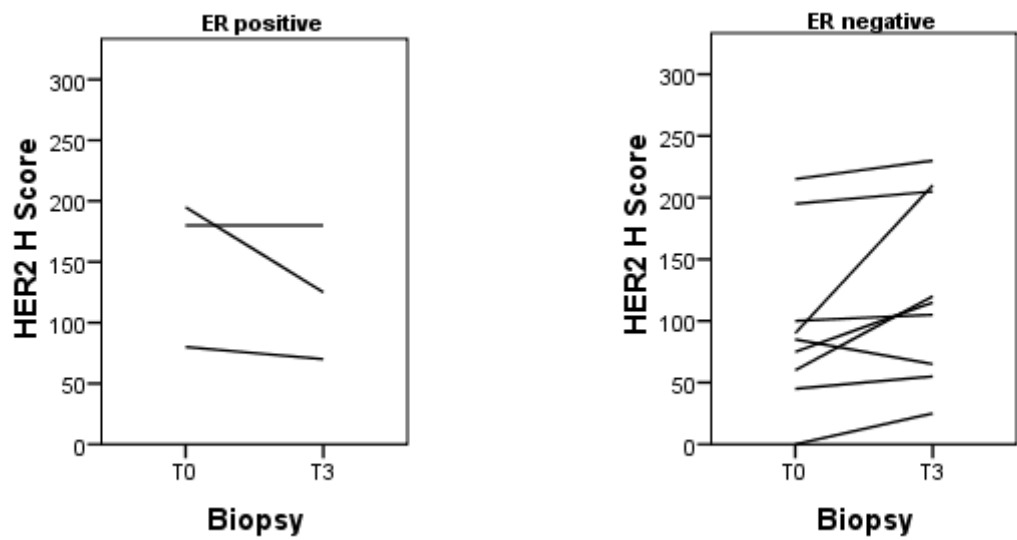


Figure 72: Changes in HER2 Expression from Pre-treatment (T0) to Progression (T3)

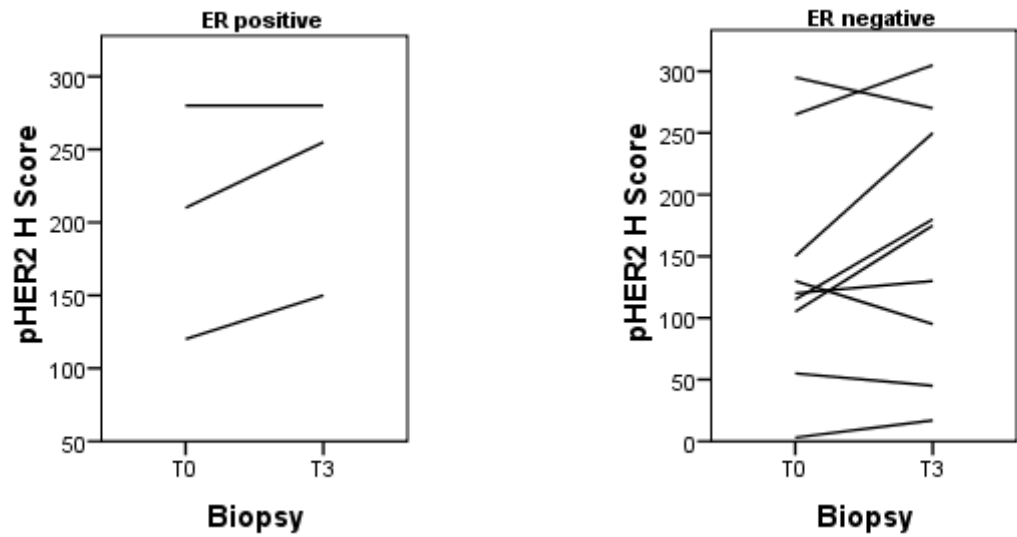


Figure 73: Changes in pHER2 Expression from Pre-treatment (T0) to Progression (T3)

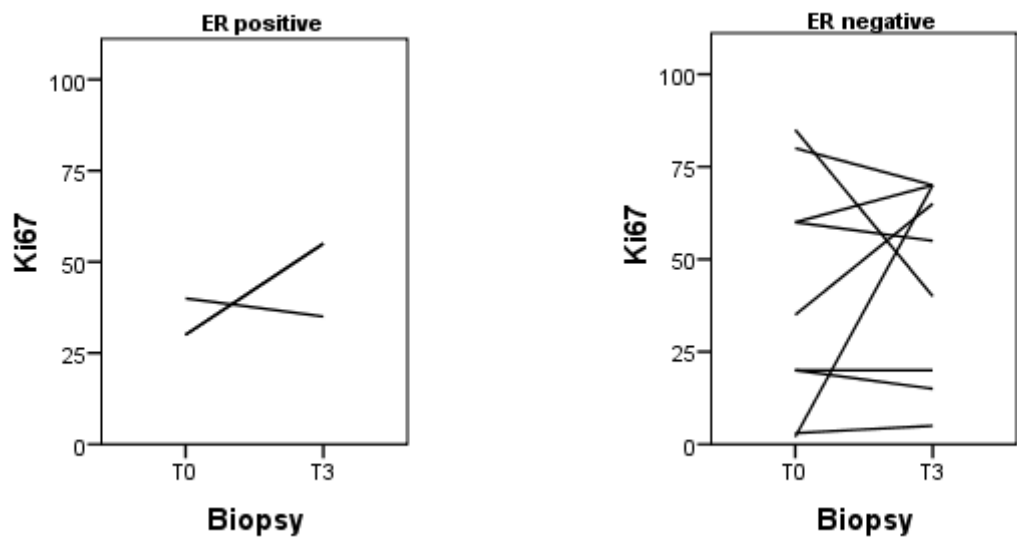


Figure 74: Changes in Ki67 Expression from Pre-treatment (T0) to Progression (T3)

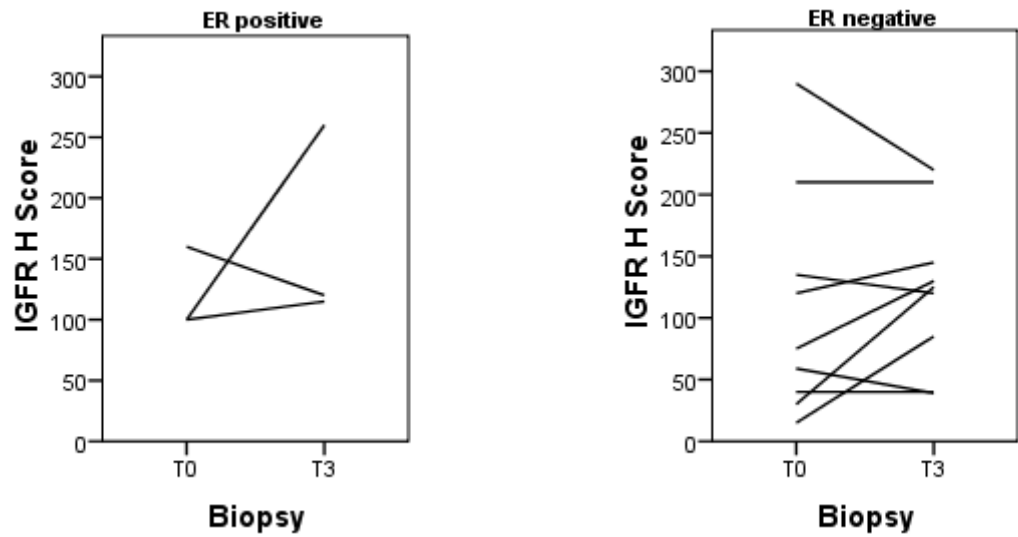


Figure 75: Changes in IGFR Expression from Pre-treatment (T0) to Progression (T3)

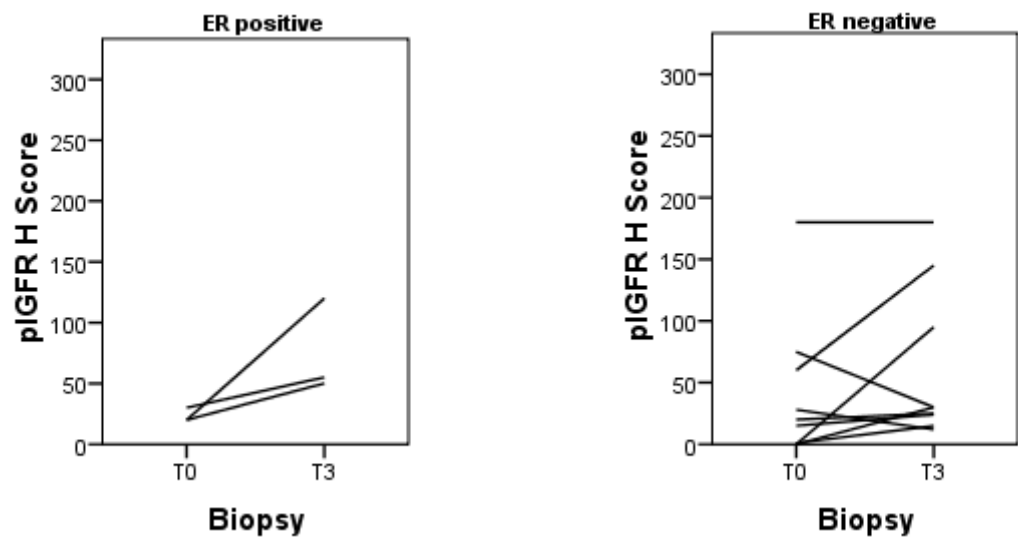


Figure 76: Changes in pIGFR Expression from Pre-treatment (T0) to Progression (T3)

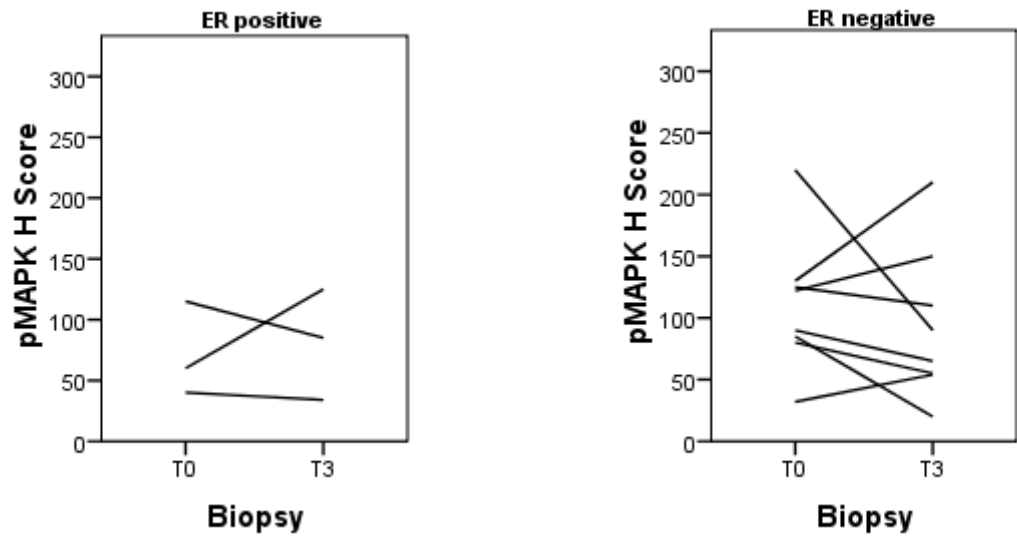


Figure 77: Changes in pMAPK Expression from Pre-treatment (T0) to Progression (T3)

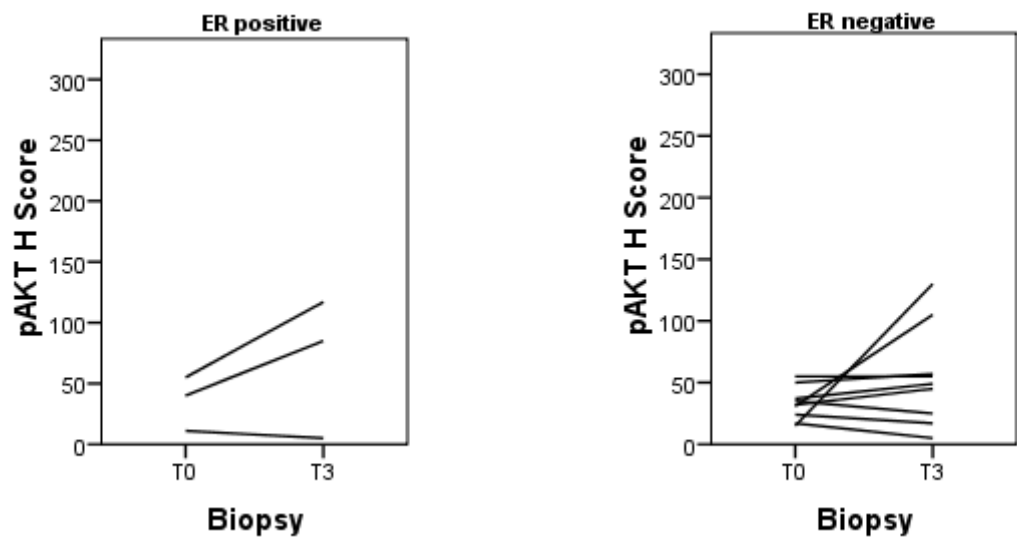


Figure 78: Changes in pAKT Expression from Pre-treatment (T0) to Progression (T3)

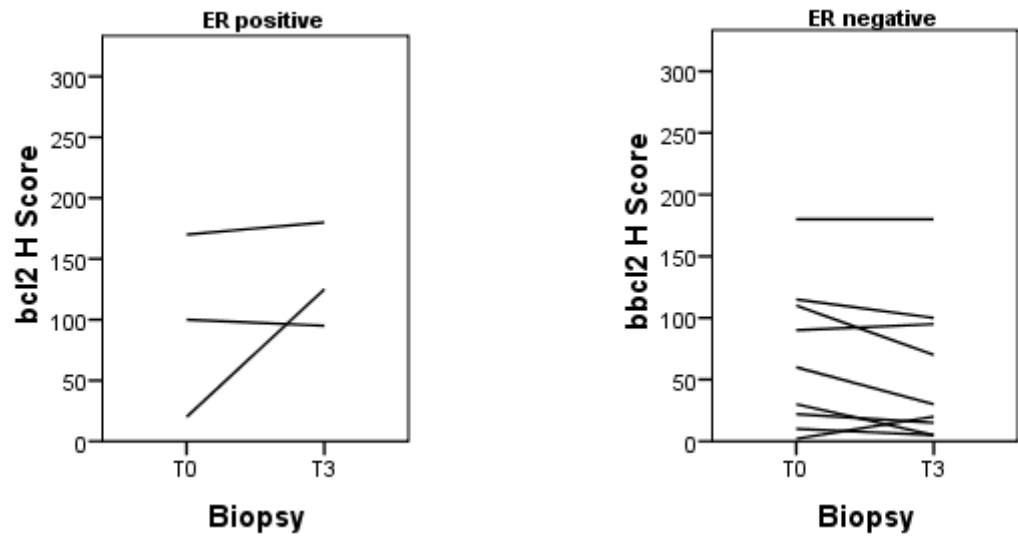


Figure 79: Changes in bcl2 Expression from Pre-treatment (T0) to Progression (T3)

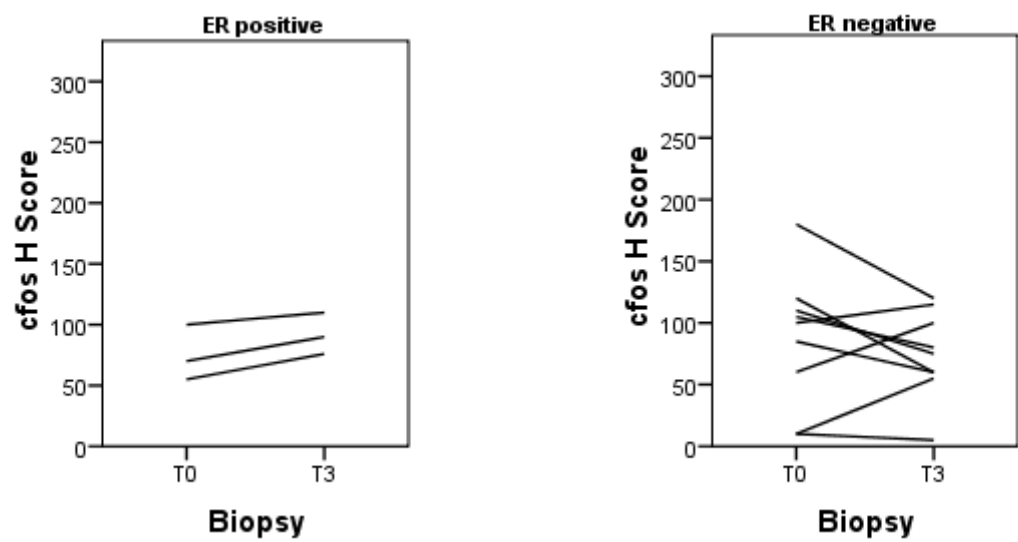


Figure 80: Changes in cfos Expression from Pre-treatment (T0) to Progression (T3)



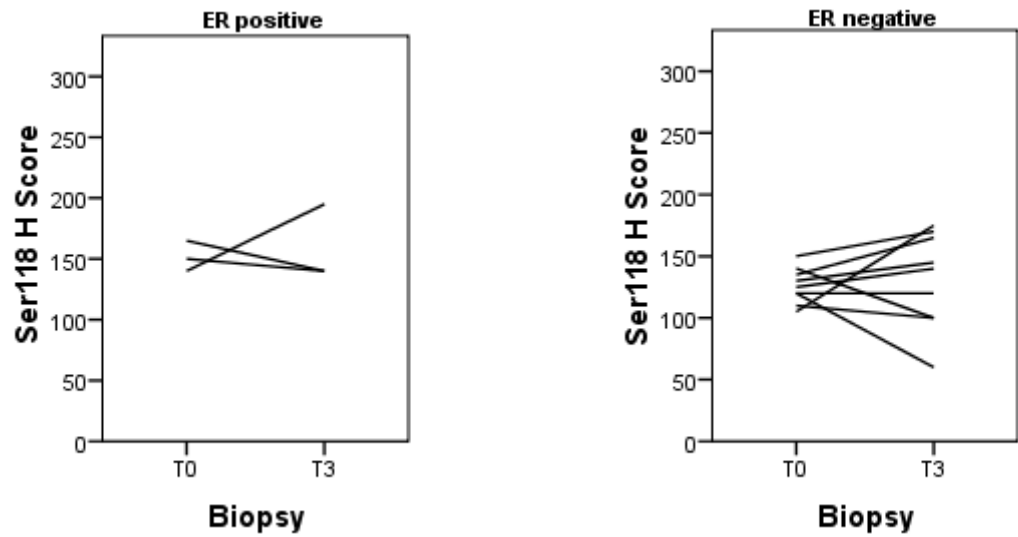


Figure 81: Changes in pSer118 Expression from Pre-treatment (T0) to Progression (T3)

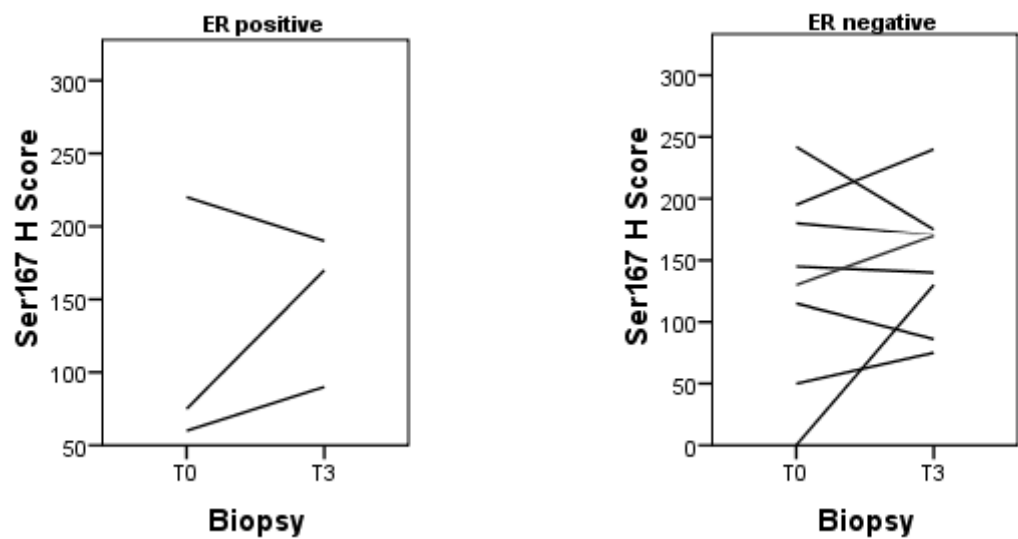


Figure 82: Changes in pSer167 Expression from Pre-treatment (T0) to Progression (T3)

## 11 Discussion

### *11.1 In Vivo Xenografts*

When this trial was being designed it was one of the first to study gefitinib in breast cancer and as such there was little efficacy data available. There were concerns regarding the potential agonistic effects of tamoxifen at the development of resistance, leading to increased gene transcription of growth-promoting proteins, including amphiregulin and IGFs [276, 277]. In breast cancer tissues tamoxifen acts mainly as an oestrogen antagonist however its agonist properties are widely reported in endometrium and bone [278-281]. In addition some human breast cancers that become tamoxifen resistant can exhibit regression on tamoxifen withdrawal further supportive of a tamoxifen mediated growth pathway [282]. Therefore, it was decided to not continue patients on tamoxifen when gefitinib was prescribed in this phase II study. This posed the question as to how much of the gefitinib growth inhibitory effect in the ER-positive/TAM-R patients might be due to tamoxifen withdrawal or in fact due to the indolent nature of some hormone sensitive tumors. To address this issue a mouse model was developed to study gefitinib sensitivity in acquired tamoxifen resistant MCF7 xenografts which expressed high levels of EGFR as in the in vitro model [158]. This is the first time xenografts have been produced from this particular acquired TAMR cell line, where previous xenograft studies have used a HER2 transfected, intrinsically tamoxifen resistant MCF7 cell line which is again gefitinib sensitive [108]. In contrast to the clinical trial, the in-vivo model studies allowed us to accurately determine the effect of gefitinib on growth of the tamoxifen resistant phenotype in the

presence of continued tamoxifen administration, mirroring the in vitro studies [133]. We randomized these xenografts to treatment with tamoxifen versus tamoxifen + gefitinib and were able to demonstrate statistically significant growth inhibition with EGFR-TKI blockade ( $p=0.039$ ) and a reduction in Ki67 ( $p=0.068$ ) confirming a gefitinib effect which is independent of tamoxifen withdrawal.

The study using in house wild type tamoxifen sensitive xenografts was devised to investigate the combination of gefitinib and tamoxifen vs. each agent alone. In cell line studies the combination of tamoxifen and gefitinib co treatment exhibited superior cell kill by inducing apoptosis and inhibiting proliferation. EGFR induction was blocked and MAPK activation abrogated leading to the prevention of EGFR mediated resistance [133]. Our hypothesis was that combining gefitinib and tamoxifen would delay the development of resistance and could prove more effective in treating oestrogen receptor positive breast cancer.

Unfortunately the study had to be abandoned. The initial dose of tamoxifen administered was only 3mg per kg per day which was probably suboptimal. We know that low dose tamoxifen can have a growth promoting or agonistic, effect. We were unable to demonstrate any tamoxifen effect on the xenografts and consequently the tamoxifen dose was increased to 10mg/kg/day at day 41. By this time the tumours had reached such a size that the animals had to be terminated at day 53. Had the initial dose of tamoxifen been correct it is probable that we would have seen delayed development of tamoxifen resistance in the face of EGFR blockade, as in the model system.

## ***11.2 Tolerability and Efficacy***

Gefitinib at 500 mg daily was generally well tolerated by patients with predictable, dose dependent side effects which resolved with dose interruption, dose reduction or cessation of therapy. Gefitinib targets the tyrosine kinase activity of EGFR receptors and as these are expressed in skin and the gut mucosa [283, 284] it is not surprising that the predominant side effects were skin dryness, acneiform skin rash, diarrhoea, nausea/vomiting, lethargy and alopecia in that order. The majority of adverse events were CTC Grades I and II. This is in line with several previous studies [251, 254] although in contrast to some reports the degree of skin rash was not associated with the quality of gefitinib response. The worst facial rashes were generally seen in ER negative non responders whilst the ER positive responders tended to have very much less florid reactions. As a result of these side effects, 17 patients had a dose reduction to 250mg and 5 patients were withdrawn due to side effects. During the study, data emerged from the lung cancer gefitinib trials [258, 285] that the lower dose of 250mg per day had shown comparable efficacy and so patients who developed significant side effects were dose reduced with less concerns re efficacy of the lower dose as the study progressed. The initial loading dose of 1000mg which appeared to be associated with early onset of side effects in some patients was also discontinued during the course of the study in view of the dose results in the lung cancer trials.

The clinical response rates in phase 2 trials of EGFR-tyrosine kinase inhibitors have been variable but generally disappointing to date. In breast cancer two small series with gefitinib have shown much lower objective response and clinical benefit rates than seen in one of the subgroups of this current study,

although the previously reported studies were in heavily pre-treated patients. Baselga et al studied 34 patients and had a median TTP of 8 weeks with a 13% CB rate. One patient achieved an objective response lasting more than 6 months and 3 patients had stable disease at 6 months [283]. Albain et al treated 63 patients. One patient achieved a partial response, and two patients had stable disease for more than 6 months, for a clinical benefit rate of 5% [258]. The Australian Clinical Trials Group enrolled 66 women with advanced breast cancer: 39 whose breast cancers had stopped responding to hormone therapy (tamoxifen followed by an aromatase inhibitor) and 27 whose tumours were ER-negative and PGR-negative. They found no gefitinib responses after 28 weeks of treatment.[286] A further phase II study of gefitinib in taxane and anthracycline resistant metastatic breast cancer recruited 58 patients who received 500mg per day until disease progression. Only one patient (1.7%) had objective partial tumor response of her liver and pleural metastasis. Fifty-seven patients (98.3%) were non-responders with 52 patients (89.7%) having progressive disease [287].

Further EGFR tyrosine kinase inhibitor studies are ongoing, but to date, a phase II trial of erlotinib (as monotherapy of 150 mg/day) in heavily pre treated locally advanced or metastatic breast cancer again exhibited only modest responses. Winer and colleagues defined two cohorts of patients treated with erlotinib. Cohort 1 had 47 patients with disease progression after multiple therapies, including anthracyclines, taxanes, and capecitabine (Xeloda); cohort 2 had 22 patients with disease progression after first-line therapy. Forty percent of the patients had received prior trastuzumab therapy. Cohort 1 had 1 PR (23 weeks) and 6 SD (all >12 weeks). Cohort 2 ( $n = 22$ ) had 1 PR (16+ weeks) and

2 SD (both >8 weeks). The most common side effects were grade 1 and 2 skin rashes (78%) and diarrhoea (59%) [286].

Further investigation into combining erlotinib with convention chemotherapy for NSCLC has again failed to show a benefit. In a large multicentre trial 1,172 patients received erlotinib (150 mg/d) or placebo, combined with up to six 21-day cycles of chemotherapy (gemcitabine 1,250 mg/m<sup>2</sup> on days 1 and 8 and cisplatin 80 mg/m<sup>2</sup> on day 1). There were no differences in overall survival or TTP, between treatment arms [261]. A follow on study TRIBUTE was a multicenter, randomized, double-blind phase III trial of TARCEVA (erlotinib) 150mg po od plus standard carboplatin and paclitaxel vs. chemotherapy alone for the first-line treatment of advanced NSCLC. TRIBUTE assessed 1059 patients but showed no difference in survival as the primary endpoint of the study.

In vitro studies with lapatinib have indicated that this dual EGFR and HER2 TKI may have promising prospects in several solid cancers including breast [288]. In vitro the combination of lapatinib + oestrogen deprivation was reported to be effective in both wild type and endocrine resistant cells [289]. A Phase I clinical trial has demonstrated activity in heavily pre-treated EGFR and HER2 overexpressing metastatic cancers [290]. In the 67 patients treated the most common adverse events were diarrhoea and rash. The most common toxicities seen with lapatinib were similar to those seen with gefitinib: in sixty-seven patients with metastatic solid tumors treated with lapatinib, the most frequently reported drug-related adverse events were diarrhea (42%) and rash (31%). Five grade 3 drug-related toxicities (gastrointestinal events and rash) were experienced by four patients [288]. There were no reported cases of

interstitial pneumonitis or cardiac dysfunction (unlike other HER2 targeting therapies). Responses were seen in 28 patients including 4 trastuzumab resistant breast cancer patients. Another Phase I trial recruited 48 patients with metastatic breast cancer; there were 16 cases of clinical benefit and 6 objective responses. A subsequent phase II trial has demonstrated that lapatinib has activity as a first line treatment for HER2 overexpressing locally advanced and metastatic breast cancer [291, 292]. A phase III randomised double blinded trial of lapatinib + placebo vs. lapatinib + letrozole in ER positive treatment naïve metastatic breast cancer (n= 1286) has shown a benefit in RR and progression free survival in the HER2 + subpopulation. There was no significant effect from the addition of lapatinib in the much larger HER2-ve subpopulation suggesting that lapatinib acts at least in large part through the inhibition of the TK on the HER2 receptor.

We know that although breast cancer treatments can be used sequentially, response rates in general fall as patients are exposed to more therapies. Mean TTPs are longer for first line therapy compared with second line therapy. One potential explanation for the poor response rates and short TTP seen with gefitinib in the above studies is that they are a reflection of the fact that the patients had received multiple treatments for their metastatic breast cancer prior to being exposed to gefitinib. The 54 patients in the study reported in this thesis were relatively treatment naïve in that they had been exposed to no more than one previous treatment for breast cancer. In this group we saw a more encouraging average clinical benefit rate of ~30%, although interestingly the vast majority of the clinical benefit was restricted to the ER positive, acquired

tamoxifen resistant group compared to the ER negative patient group despite the fact that the latter group were also relatively treatment naive.

In vitro studies have demonstrated a significant inhibitory effect of gefitinib in some ER negative breast cancer cell lines and in normal proliferating breast cells, indicating a possible role of EGFR tyrosine kinase inhibitors in the treatment and prevention of ER negative breast cancer [275, 293]. However, in this study there were only a small number of patients who exhibited steady state disease during gefitinib treatment in the ER negative subpopulation. (CB rate 12%, n = 3.) During the writing of this thesis it had become established that gefitinib worked better in patient groups with certain features – i.e. non-smokers compared to smokers, Asian compared to Caucasian and women compared to men [294] . Furthermore there were two initial publications [295, 296] which reported the presence of somatic mutations in the TK domain of the EGFR receptor which appeared to increase the sensitivity of the mutant receptor to gefitinib. Further work confirmed these findings and indeed a recent paper has reported that gefitinib alone is significantly more effective than standard chemotherapy in mutation positive tumours but the reverse is true in mutation negative lung cancers [297]. These findings highlight the importance of identifying the appropriate population to treat and predictive biological marker(s) in order to develop biological therapies as intelligently and swiftly as possible. In line with this study, ER negativity has been recently shown to be a poor indicator of response to gefitinib plus docetaxel as first line therapy [292] in metastatic breast cancer. This indicates that there may be other dominant mitogenic signalling routes in ER negative cells, potentially involving alternative classes of growth factor receptors together with their associated



ligands. For example, the availability of such elements prior or subsequent to EGFR blockade could provide a mechanism whereby cells might reduce / circumvent EGFR inhibition. Indeed, some studies have demonstrated that insulin-like growth factor-1 (IGF1-R), basic fibroblast growth factor (FGF) and heregulins can all override the growth inhibitory effects of EGFR blockade in cancer cells [298].

The vast majority of gefitinib responses seen in this study were in the ER positive acquired tamoxifen resistant subgroup. We were able to demonstrate a CB rate of 56.5 % (n = 13) in this group, the majority of whom had prolonged periods of remission. These encouraging observations are in line with in vitro models of acquired tamoxifen resistant MCF-7 and T47D cells [132]; which are also growth inhibited by gefitinib. The data are also in line with previously reported xenograft studies using an MCF7 HER2 transfected, tamoxifen resistant breast cancer cell line [108]. In-vitro studies of gefitinib treatment of TAMR cells have reported a reduction in EGFR phosphorylation and downstream MAPK signalling and thereby growth. Similar findings were also detected in a proportion of acquired tamoxifen resistant CB patients providing evidence in this study indicating that gefitinib is acting via depleting EGFR receptor signalling in these responsive patients as in the model system.

However, Baselga's group [254] were able to demonstrate more substantial reductions in activated MAPK in the skin of patients on gefitinib than we observed in the present study. Indeed, since decreases in activated EGFR were not universal in patients with CB in this trial, a non classical gefitinib response mechanism may exist in some patients. In summary, trials in metastatic breast cancer have shown no efficacy in heavily pre-treated patients although there is

no evidence that these tumours expressed EGFR. Efficacy was seen in some clinical studies in TAMR tumours where some EGFR expression was required but the level of EGFR expression was not predictive. Where the biology of the tumours was characterized the best clinical responses were seen in ER+ PgR+ breast cancers. In the randomised Phase 2 clinical trials there was no significant effect overall when gefitinib was added to tamoxifen but retrospective analysis has suggested gefitinib may have been more effect in a hormone naive subgroup. In contrast a smaller randomized Phase 2 study of anastrozole +/- gefitinib reported a significant benefit in favour of the combination. However this was a small study and there remain some outstanding questions regarding these results.

### **11.3 Predictors of Response**

Acquired tamoxifen-resistant cells in vitro retain ER and there is evidence of productive cross-talk between the ER and the EGFR that drives tumour cell growth [265]. This is in keeping with the strong association we observed in the current study between clinical benefit with gefitinib and ER positivity, the strongest predictive biomarker discriminated. The BCIRG 103 study was a presurgical study of 250mg daily gefitinib comparing core biopsies with operative samples in 59 patients [118]. The researchers identified a subset of ER positive, PgR weak breast cancers which are more likely to be driven by growth factor signalling mediated growth. This is not the case in our study with the majority of responders in the TAMR group being both strongly ER and PgR positive. Although ER positivity and lack of obvious EGFR overexpression was a strong predictor of response, all responders expressed some level of EGFR (median HScore=30, range=10–65) which is further supporting evidence that gefitinib is acting via the EGFR receptor. This is in line with previous studies which have indicated that it is the presence of EGFR rather than the magnitude of expression that predicts response [239]. Specific mutations in the EGFR receptor have been implicated in the response to gefitinib in NSCLC but not in breast cancer to date [299]. These were not assessed in this study but it would be possible to reassess the samples for these additional possible predictors of response. The levels of EGFR phosphorylation detected were not predictive of response. Efficacy of gefitinib was also independent of EGFR overexpression in the IDEAL non-small cell lung carcinoma (NSCLC) trials [300]. In our study high levels of EGFR expression were significantly associated with a higher incidence of progressive disease

and a shorter TTP. Pre-surgical and neoadjuvant studies do not provide a consistent picture of what type of tumours are sensitive to gefitinib or what tissue markers reflect or predict the biological effects of gefitinib. Furthermore biological studies do not in the majority of cases seem to link well with the clinical studies. One consistent result however appeared to be that EGFR expression is required to see any biological activity.

HER2 expression has been associated with poor prognosis and systemic treatment failure [143, 301]. However in vitro and in vivo NSCLC HER2 expression had no bearing on gefitinib response [302, 303]. HER2 and its activity were also not predictive of response/failure in this clinical study. In the ER negative, gefitinib resistant phenotype the frequency of HER2 expression was high. However, in the ER positive tamoxifen resistant phenotype levels of HER2 were higher than would be expected from a relatively treatment naïve population of ER positive breast cancers yet this did not preclude a response to gefitinib, in keeping with tamoxifen resistance observations experimentally [108]. In the BIG 1-98 adjuvant trial overexpression of HER2 was associated with a poorer outcome in ER positive tumours whether treated by tamoxifen or letrozole [304]. However the findings in the BIG1-98 study and this study are not consistent.

In vitro studies have identified a NSCLC cell line which expresses low EGFR and high levels of HER2, mirroring our TAMR patient group. These HER2 transfected cells are sensitised to gefitinib and exhibited marked growth inhibition [305]. It may be that the high levels of HER2 expressed in our TAMR group were exerting a similar effect to this in vitro study.

High levels of activated AKT expression have been associated with a more aggressive phenotype in vitro. Cell lines which have developed tamoxifen resistance and are sensitive to gefitinib have high levels of activated AKT-1 [132, 176]. The expression of activated AKT has been implicated in the development of multidrug resistance [182]. Small studies in metastatic patients have confirmed that the expression of activated AKT and HER2 is an indicator of poor response to endocrine therapy in this setting [183]. In this study baseline activated AKT expression had no bearing on response. As discussed anti-oestrogen resistant cell lines have increased levels of activated AKT but we did not see significantly higher levels of AKT in our responders, which were almost exclusively ER positive TAMR. In this study baseline activated AKT expression was not associated with response and there was no significant downregulation of expression with gefitinib, however high levels of activated AKT have been shown in vitro to confer gefitinib resistance raising the question of combining therapies to block both targets [306].

Long term oestrogen deprived cell lines express elevated levels of activated MAPK and use these pathways for cell proliferation in the absence of oestrogen as a growth promoter [166-168]. We were unable to detect any difference in MAPK expression at baseline in our two patient groups. In clinical cancer specimens strong nuclear MAPK staining has been significantly associated with poor response, shorter TTP and decreased overall survival [273].

A high level of Ki67 expression is associated with highly proliferative tumours and endocrine insensitivity [200]. High Ki67 expression is associated with ER negativity, EGFR positivity and decreased patient survival [203-206]. However

in this study baseline proliferation as measured by Ki67 had no influence on gefitinib response. This may be that higher levels of Ki67 expression were induced at the development of tamoxifen resistance and so baseline expression in the ER positive TAMR patients approached the levels of the ER negative group (which would be expected to be high).

## **11.4 Biomarker Changes on Gefitinib**

### **11.4.1 ER, pSer118 and pSer167**

There was little gefitinib effect on the expression of ER after 8 weeks on treatment in the two subgroups, with both having small decreases. At the 6 month time point the ER positive patients there was again no discernable effect. At the development of resistance the ER positive samples had a mean change from baseline to increase ER expression whilst again the ER negative patients had no obvious change. Ser118 expression was unaffected by gefitinib administration at 8 weeks with a small increase at 6 months in the ER positive patients whilst the single ER negative patient also had a small increase. At the development of resistance pSer118 levels were decreased in both groups. This models some cell line studies where at the development of resistance to aromatase inhibitors and in the face of profound oestrogen deprivation by fulvestrant, ER levels increase in the face of decreased levels of the activated Ser118 suggesting ligand independent growth promotion [309], however detection of pSer118 has been shown to be an indicator of an intact ligand-dependent ER- $\alpha$  in breast tumors in vivo and to predict responsiveness to endocrine therapy in particular tamoxifen [310, 311]. The absolute decrease in pSer118 has recently been shown to mirror tumour response to endocrine therapy but we demonstrated no association with gefitinib [312]. pSer118 expression is driven in part by the MAPK pathway and as there were increases seen in this marker it is surprising that we did not see similar increases in pSer118 at this time point. We did, however observe an increase at 6 months in line with a parallel increase in pMAPK.

In the first 8 weeks of treatment the ER negative patients exhibited a large mean increase in pSer167 expression. At 6 months there were some small increases in the mean expression in the ER positive group and the ER negative patient had a similar increase. Again at the development of resistance the ER negative group had large mean increases in expression vs. baseline, whereas the ER positive patients revealed no mean change

In vitro studies have shown that the phosphorylation of Ser167 can be mediated by overexpression of EGFR and tamoxifen resistance can be restored by AKT inhibition [311]. However conflicting reports suggest that expression of pSer167 confers better survival after relapse in metastatic breast cancer patients [112]. Some large increases in activated AKT were seen at the development of resistance in the ER negative group and as Ser167 is activated by AKT this could provide a mechanism for increased Ser167 levels.

#### **11.4.2 PgR**

Gefitinib had little effect on PgR expression in the early treatment biopsies vs. baseline in both patient groups with ER positive patients exhibiting a small fall from baseline but ER negative patients showing no change in their already very low levels. After 6 months the ER positive patients showed an increase in their PgR expression. At the development of resistance the ER positive samples had a mean change from baseline to increase PgR expression. As PgR is induced by a functioning ER this could indicate re-activation of the ER via cross-talk with the growth factor receptor pathways. PgR can induce changes in the pMAPK pathway and may in part lead to the elevated levels of pMAPK seen at this time point as in the model system of endocrine and TKI resistance.



### **11.4.3 EGFR and pEGFR**

In the first 8 weeks of gefitinib administration EGFR increases were more often seen in the ER negative population which had initially expressed higher levels. This has been observed in de novo gefitinib resistant cancer models in vitro. These tumours were also more likely to show early progression than their ER positive counterparts. However pEGFR expression fell further in this subgroup which may indicate growth driven by an EGFR-kinase independent manner via crosstalk with other growth factor receptors such as HER2 and IGFR. At 6 months on treatment the ER positive group we observed a modest increase in expression of EGFR but no change in the levels of pEGFR. At the development of resistance both groups had a mean increase in EGFR from baseline but a mean decrease in its activated form. This is in accordance with the predicted mechanism of action of gefitinib and indicates that in this subset the drug is “hitting the target”. However decreases in EGFR activation were not universal across the whole patient group

Since the design of this trial data has emerged which suggests that response to gefitinib in the NSCLC trials is associated with EGFR gene mutations, specifically on exons 18 – 21 [313]. However these mutations have not been seen in primary or metastatic breast cancer. Despite the lack of efficacy seen when combining gefitinib with conventional chemotherapy in NSCLC there are further trials ongoing, targeting those groups thought most likely to benefit. The IPASS lung cancer trial is currently recruiting and is a randomised multicentre Phase III trial based in Asia. It aims to compare gefitinib with combination chemotherapy (carboplatin and paclitaxel) as first line therapy in patients who have never (or only lightly) smoked. Its exploratory endpoint is

progression free survival and it will also perform biomarker analysis on archival tumour tissue.

Whilst the induction of the downstream MAPK and AKT pathways is seen at resistance there is growing evidence that EGF-related ligands (i.e., EGF, amphiregulin, transforming growth factor- $\alpha$ , beta-cellulin, epiregulin and neuregulins) may be upregulated with gefitinib treatment. Breast cancer cells intrinsically resistant to gefitinib markedly up-regulate the expression of genes codifying for EGF-specific ligands. In addition loss of EGFR function affects the nucleo-cytoplasmic trafficking of EGF-related ligands indicating an “intracrine” feedback mechanism which is independent of the expression or activation of HER2 [314]. These EGF-related ligands were not assessed in this study and it would be interesting to revisit the tumour samples to see if this phenomenon also occurred in this group of breast cancers

#### **11.4.4 HER2 and pHER2**

In the first 8 weeks of treatment HER2 expression was reduced across both patient groups. pHER2 however was increased particularly in the ER negative samples who in the main were the early progressors. At the 6 month time point the ER positive tumours had a decrease in HER2 expression but an increase in HER2 activation. At the development of gefitinib resistance we demonstrated a mean decrease in HER2 expression in the ER positive group with a corresponding increase in the ER negative tumours. Interestingly the mean change from baseline in pHER2 was an increase.

This is in line with the observations in the in vitro studies into the effects of gefitinib on the growth of breast cancer cell lines expressing different levels of

EGFR and HER2 receptors. The heterodimerisation of these receptors was studied in HER2-overexpressing BT-474 breast cancer cells under basal and ligand-stimulated conditions. Gefitinib was found to inhibit the growth of these HER2 overexpressing cell lines. Because gefitinib does not inhibit the HER2 tyrosine kinase in vitro, and because heregulin is a ligand that activates HER2 by binding to HER3 and HER4, it was suggested that gefitinib inhibits the growth of HER2-overexpressing breast cancer cells, possibly by sequestration of HER2 and HER3 receptors with the EGFR inducing the formation of inactive unphosphorylated EGFR/HER2 and EGFR/HER3 heterodimers [138]. There were some increases in pHER2 expression seen at resistance and this would be consistent with the development of a more aggressive phenotype and has been demonstrated in prostate cancer cell lines which show elevated levels of HER2 after prolonged gefitinib exposure [315].

#### **11.4.5      Ki67**

While there was no difference in proliferation between the responders and the early progressors prior to gefitinib treatment, we were able to demonstrate a significant fall in tumour Ki67 levels during early treatment with gefitinib in the responding patient group and hence surmised that Ki67 level attained at T1 was predictive of outcome. Declines in Ki67 positivity of >10% were common in responders, but were rarely observed in the early progressors. Pre-surgical studies with gefitinib are difficult to interpret with no dominant biological hypothesis emerging from the data. In a small study in patients with dual EGFR+, ER+ primary breast cancers those randomised to gefitinib +/- aromatase inhibitor anastrozole demonstrated that the combination regime lead to a greater decrease in Ki67 levels and better tumour response than gefitinib

alone. In addition the study also reported that gefitinib as monotherapy or in combination with anastrozole also reduced levels of pEGFR, pER and MAPK [214]. In this double-blind, placebo-controlled randomised trial of 56 postmenopausal patients with ER-positive and EGFR-positive primary breast cancer, 27 women received gefitinib plus anastrozole and 29 women gefitinib alone for 4-6 weeks before surgery. The combination therapy had a greater effect on Ki67 and produced reductions in tumour size, raising the possibility of combining these agents. A further pre-surgical study confirmed the requirement for EGFR expression in the tumour but suggested that EGFR inhibition may be of more effective in ER+ve PgR-ve breast cancers [316]. However another large trial recently reported no significant difference in Ki67 expression with the above combination. This was a phase II, randomised, parallel group, double blind and placebo-controlled multicentre study comparing the efficacy and safety of anastrozole (1 mg daily) and placebo versus anastrozole (1 mg daily) and gefitinib (250 mg daily) when given to postmenopausal women with ER+ EGFR- breast cancer for up to 16 weeks. 270 postmenopausal women, with oestrogen receptor (ER) and/or progesterone receptor (PGR) positive newly diagnosed non inflammatory invasive breast cancer were enrolled in the study and 206 were randomised to treatment. No significant differences were seen in tumour response or Ki67 expression between the two groups. [215] However there was a numerical but not statistically significant increase in response rates in favour of the anastrozole alone group [317].

This change in Ki67 staining is in line with several studies which examine response to other types of treatment for breast cancer [195, 202, 208, 318] and

is a reflection of decreased cell proliferation with therapy. A matched analysis at T1 revealed that five of the patients achieving CB showed a >10% T0-T1 fall in activated EGFR and further biomarker examination in these patients revealed decreases in phosphorylation of the downstream signalling element MAPK and Ki67 proliferative capacity. The 2 CB patients with the highest levels of phosphorylated EGFR at T0 both showed substantial falls in receptor activity with gefitinib. A reduction in Ki67 levels in this study was also seen (alongside significant longer-term growth inhibitory effects) in the acquired tamoxifen resistant xenografts after 14 days of treatment treated with gefitinib in the presence of tamoxifen compared to those treated with tamoxifen alone. The Ki67 difference approached statistical significance ( $p = 0.068$ ) – despite the fact that Ki67 was assessed in only 5 mice per group. This drop in Ki67 was predictive of a significant growth inhibitory effect of gefitinib ( $p < 0.05$ ) seen during the course of this experiment. Recent studies including the BCIRG 103 study have also examined early biological marker changes in human breast cancer tissue including Ki67 and seen a reduction in proliferation [118].

#### **11.4.6 IGFR and pIGFR**

After 8 weeks of gefitinib there was a small decrease in IGFR and pIGFR in the ER positive group. The ER negative group had small increases in these markers.

At 6 months the ER positive patients exhibited increases in IGFR and its activated form. At the development of resistance there were increases in IGFR and pIGFR in both subgroups but particularly in the ER positive patients. In cell culture, acquired tamoxifen resistance is associated with functional IGFR signalling which interacts with the EGFR pathway to enhance its promotion of cell growth [158]. Thus in such cells, response to gefitinib is not precluded by presence of IGFR. IGFR blockade has been shown to restore gefitinib sensitivity in colon cancer cell lines [307]. However signalling via alternative receptors and EGFR phosphorylation mediated in an EGFR kinase-independent manner by such receptors, including IGFR (readily detectable in all the breast cancers in this study) as well as heterodimerisation with other HER family members, have been implicated in anti-EGFR resistance in vitro in several cancer types. In the TAMR model elevated IGFR signalling ultimately drives acquired gefitinib resistance that is emergent during prolonged drug exposure [311]. Continuous exposure of EGFR-positive TAMR MCF7 breast cancer cells to 1 microM gefitinib results in a sustained growth inhibition of approximately 90% for 4 months before the surviving cells resume proliferation. A stable gefitinib-resistant subline (TAM/TKI-R) has been established after a further 2 months and this has no detectable basal phosphorylated EGFR activity. Compared with the parental TAMR cells, the TAM/TKI-R cells demonstrate elevated levels of activated IGFR and AKT.

Such observations in total indicate intrinsic IGFR levels may not discriminate between initial gefitinib response and failure, and in keeping with this in the present study the level of IGFR expression and its activity pre-treatment were not predictive.

#### **11.4.7 pMAPK**

After 8 weeks on gefitinib the ER positive patients had small decreases in pMAPK with a somewhat greater change from baseline in the ER negative group. At 6 months the ER positive patients showed mean increases in pMAPK and the other downstream effectors. At the development of resistance the ER positive patients had increases in pMAPK whilst the ER negative group had a mean decrease from baseline. In vitro studies have seen that cells which have low sensitivity to gefitinib express high levels of MAPK [315] and that inhibition of the MAPK pathway in addition to gefitinib administration causes marked apoptosis and cell death. A gefitinib insensitive cell line had its sensitivity restored when a MAPK blocking agent (PD98059) was administered, producing a significant increase in the levels of apoptosis [319]. This gives support to the theory that the MAPK pathway is involved in gefitinib resistance and raises the possibility of combining anti MAPK agents with gefitinib to delay the development of resistance. The MAPK pathway has been implicated in the development of endocrine resistance via activation of the Ser118 site on the oestrogen receptor [320, 321].

#### **11.4.8 pAKT**

There was a greater fall in pAKT levels in the ER negative group during the first 8 weeks of treatment. At 6 months the ER positive patients had increase levels of pAKT. At the development of resistance we observed some increases in pAKT in the ER positive group. The ER negative group had a large mean changes to increase pAKT

This is in accordance with cell line studies which have implicated AKT in the development of endocrine resistance and a more aggressive phenotype with prolonged cell survival [176, 184]. In human ER positive human breast cancer specimens AKT activation is associated with activation of both HER2 and ER promoting tamoxifen resistance [109].

#### **11.4.9 Cfos**

Fos protein expression was upregulated from baseline to 6 months and the development of resistance in the ER positive group. Elevated Fos expression has been shown to correlate with endocrine resistance where decreases in Fos, proliferation and cellularity at 6 months predicted better responses [198].

However the nuclear transcription factor Fos is inducible by both steroid hormones and growth factors and is a potential point of interaction between steroid hormone- and growth factor-directed pathways. Sustained elevated levels of Fos expression are significantly associated with further factors, notably peptide growth factors and their receptors (e.g., EGFR, TGF alpha) [196]. In light of the new evidence that the expression of TGF alpha and other EGFR ligands are upregulated in acquired and de novo TKI resistance it may be that elevated fos protein precedes and induces the transcription for these ligands. That would offer a mechanism for increased levels of fos protein at the



6 month time point in this patient group who went on to develop acquired TKI resistance.

#### **11.4.10 Bcl2**

In the early treatment period there were no striking differences in bcl2 change from baseline in either group. There was a small rise in both groups at 6 months vs. baseline and no obvious change from baseline at the development of resistance. As bcl2 expression is closely linked with steroid hormone expression and we observed little change in ER and PgR it is perhaps not surprising that there was also little change in this marker.

## 12 Conclusion

The process of drug development is expensive and time consuming with millions of dollars spent in the testing of new chemical entities [322, 323]. Unfortunately many agents which show initial promising activity against a particular biological target will be discarded due to concerns regarding their safety, toxicity and efficacy in humans and there is a perception that pre-clinical models may foster unrealistic expectations and many promising drugs are failing to reach their potential. In addition reporting of clinical trials may disadvantage certain drugs at an early stage in their development. The introduction of the tyrosine kinase inhibitors was met with great optimism as it was anticipated that they would become an important weapon in the fight against many types of cancer.

In the MCF7 model in vitro system prolonged exposure, of approximately 3 months, to Tamoxifen at  $10^{-7}$  M leads to development of resistance via increased EGFR and HER2 signalling. TAMR cells acquire characteristics associated with invasiveness, such as increased motility and decreased adhesion factors. EGFR and HER2 heterodimerise and activate to effectively recruit multiple downstream growth factor kinase cascades including MAPK and AKT. The activated MAPK and AKT crosstalk with the nuclear ER, via a positive feedback mechanism, to drive its activation via Ser118 and Ser167 recruitment on the ER alpha AF1 site. The activated ER then feeds back to drive further EGFR ligand expression. This establishes a self-propagating EGFR/HER2/ER dependent autocrine signalling loop that efficiently drives acquired TAMR growth. In addition to this increased levels of EGFR ligands

such as TGF alpha are detected in TAMR breast cancers at the development of resistance providing a substrate for further EGFR phosphorylation.

The expression of ER and EGFR has an inverse relationship. ER negative growth is driven via the EGFR pathway. Hence 2 groups of patients were identified who may derive benefit from an EGFR TKI. Recruitment to the study was extremely slow, particularly in the ER positive cohort who had an aromatase inhibitor as a well established next sequential therapy available as an option. In patients with ER-negative tumours the effects of gefitinib were at best modest, in contrast to the effects observed in patients with ER-positive, acquired TAMR tumours, where cell proliferation appears, at least in part, to be mediated through EGFR signalling and can be blocked with gefitinib. This observation is very exciting as it mirrors the in vitro cell model. All responders expressed EGFR to some degree and biomarker changes during gefitinib treatment confirm that the drug targets the EGFR receptor leading to decreases in the levels of activated EGFR in this group. Clinical benefit with gefitinib was most commonly seen in patients with ER/PGR-positive tumours exhibiting low EGFR expression, with proliferation changes at T1 paralleling subsequent response.

In the model system IGFR expression and activation is elevated with chronic tamoxifen administration. The activated ER also stimulates the expression of IGF II which again reactivates the IGFR. Once again this provides a positive feedback loop which facilitates EGFR signaling. At the development of gefitinib resistance there were increases in the levels of IGFR expression in line with the in vitro model. Agents which inhibit IGFR have a knock on effect on the expression of activated EGFR and are promising agents in the fight

against resistance. This biological aspect of this study was small and exploratory conceived when there was very little data available about the action of gefitinib *in vivo*. It emphasises the importance of clinical cancer tumour biology studies as it is essential to investigate how the laboratory findings translate into clinical practice. The numbers of matched tumour samples available for analysis was relatively small emphasising the difficulty of such long term studies which involve sequential biopsies. Nevertheless we have obtained some robust data which has confirmed the model system and have identified a cohort of breast cancers which appear to derive benefit from a targeted EGFR tyrosine kinase inhibitor. Immunohistochemistry has confirmed a fall in the proliferation index Ki67 and the *in vitro* hypothesis that EGFR blockade leads to decreased levels of phosphorylated EGFR in the responders is supported. We have been able to demonstrate that EGFR TKI resistance is associated with significant increases in EGFR expression and phosphorylation and with some increases in activated AKT levels raising the possibility of targeting these two receptors.

Gefitinib at the higher 500mg dose did induce side effects frequently although most patients with advanced breast cancer appeared willing to tolerate them. Reduction in the dose to 250mg was however much better tolerated throughout the patient group. This study has shown that gefitinib was effective in acquired tamoxifen resistant tumours in *in vitro* and *in vivo* models, as well as in acquired tamoxifen resistant human breast cancer in the clinic, where previous clinical studies in unselected, heavily pretreated patients had reported very little efficacy. There therefore appears to be a cohort of breast cancer patients who obtain more substantial benefit from EGFR-TKI treatment. Various *in*

*vitro* and *in vivo* models to date have previously reported that as well as effectively treating acquired tamoxifen resistance, gefitinib in combination with tamoxifen can delay, or even prevent, acquired tamoxifen resistance in breast cancer. Further studies evaluating gefitinib in combination with other endocrine agents, such as anastrozole and fulvestrant are in progress. There is an EORTC trial attempting to answer this same question, 108 patients have been enrolled. The Eastern Cooperative Oncology Group is running a randomized phase II trial of combination anastrozole plus ZD1839 and of combination fulvestrant plus ZD1839 in the treatment of 148 postmenopausal women with hormone receptor-positive metastatic breast cancer. A further study: is evaluating the combination of fulvestrant, anastrozole and gefitinib as primary systemic therapy in 40 postmenopausal women with hormone receptor-positive breast cancer. Finally 44 patients have been recruited to a single arm phase II trial evaluating the activity of gefitinib in metastatic breast cancer pre-treated with an antiestrogen and a non-steroidal aromatase inhibitor, either anastrozole or letrozole. If these clinical trials show that gefitinib can delay or prevent acquired endocrine resistance, this will provide new opportunities for extending disease control.

The current spectrum of pre-clinical models only partially reflects the true heterogeneity of breast cancer and as clinicians we must be aware of the limitations of results from these model systems. Clinical trials are still essential to the development of new generations of biological agents but traditional large clinical trials may not be the best way of evaluating agents which have a target expressed in only a minority of breast cancers. With an emphasis on individualising treatment, the full potential of new agents is may not be being

fully realized and we must strive to have a better understanding of tumour biology in order to fully assess what improvements in outcome can be expected. Large pre-surgical studies such as the POETIC trial may well be the future in investigating new agents,

## 13 Appendix

### 13.1 Trial Consent Form

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**Patient Written Informed Consent**

Study Code	1839IL/0057
Edition No.	Version 2
Date	28 <sup>th</sup> February 2003

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#### **Patient Written Informed Consent**

**Phase II trial to assess the efficacy of ZD1839 (Gefitinib™) 500mg/day in patient with breast cancer who have either failed on tamoxifen or have an oestrogen receptor negative tumour and would be considered for systemic therapy.**

You are being asked to take part in a research study. Before you decide it is important for you to understand why the research is being done, what it will involve and the possible benefits, risks and discomforts. Please take time to read the following information carefully and discuss it with your family doctor, if you wish.

#### **What is the background and purpose of the study?**

ZD1839 (GEFITINIB™) is a new drug that has shown promising activity against a number of types of cancer and has been shown to slow or stop growth in tumours. It works differently from the way chemotherapy drugs work. No benefit was obtained from adding ZD1839 (GEFITINIB™) to platinum-based

chemotherapy (gemcitabine and cisplatin or paclitaxel and carboplatin in 2 large Phase III trials in patients with advanced lung cancer (NSCLC) who had not previously received any chemotherapy.

Your doctor believes your type of breast cancer is the sort which may respond to this new drug. This is based on knowledge of two things. The first is your own cancer and the biological markers which it does or does not express and also any previous treatment you have had and whether your cancer is likely to respond to this new treatment. Secondly, from the results of experiments carried out on breast cancer cells which were inhibited by this drug, ZD1839 (GEFITINIB™), your doctor believes that your cancer is the sort which may benefit from this new treatment. In these latter experiments the growth of breast cancer cells was inhibited by ZD1839 (GEFITINIB™). It is hoped that ZD1839 (GEFITINIB™) will produce similar results in patients with breast cancer such as yourself.

Approximately 54 other subjects like yourself will take part.

### **Do I have to take part?**

It is up to you whether or not to take part. If you do decide to take part you will be given this written informed consent to sign. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive. We would only ask that you bring your decision to withdraw to the attention of your hospital doctor.

Equally we will keep you informed of any new information and if there is any clinical reason for you to withdraw from the study you will be informed of this as soon as possible.



If you do not want to take part in the study there are other options available for you which your study doctor will have discussed with you.

**What will happen to me if I take part?**

This study will run for up to 2 years. Your participating in this study may therefore last for up to 2 years. The exact time depends on when you start, how your cancer responds to the study drug and if you have any side effects.

If you choose to take part in this study you will complete a 'screening' visit to determine if you meet the study requirements. You may also have further x-rays or tests which the doctor or research nurse will explain to you.

In addition some blood will be taken for laboratory tests. Up to approximately 4 teaspoonfuls (14-21ml) of blood will be taken. A pregnancy test will also be performed on women who can have children.

Once the treatment has started you will return to see your doctor or research nurse every four weeks for study visits (during the first month only you will also be seen after two weeks). At these visits you may have a physical examination and some blood tests will be performed. Your doctor will also check how well you are tolerating the study drug (side effects). An assessment will be carried out every 12 weeks after 3 months as long as you continue to participate in the trial.

Your Study Doctor has been asked by AstraZeneca to supply if possible to take some tissue samples from your tumour. This was probably done by biopsy when your disease was first diagnosed. These samples will be used in a laboratory to investigate further how the study medication works in breast cancer. The sample may be used again in the future if new technology allows more advanced tests to be done. These tests will be for research purposes only.

The study medications must be taken as follows:

ZD1839 (GEFITINIB) – You will take the ZD1839 (GEFITINIB™) tablet(s) once a day, every day about the same time. You can take your tablet(s) with or without food. If you forget to take a dose, take the last missed dose as soon as you remember, as long as it is at least 12 hours before the next dose is due. If it is less than 12 hours to the next dose, do not take the dose you have missed.

You will take your first dose on Day 1, then every day until your breast cancer progresses or an unacceptable side-effect occurs or you withdraw consent.

If you are unable to swallow the ZD1839 (GEFITINIB™) tablets, your Study Doctor will give you special instructions for preparing the dose of study drug in water.

When you have finished taking part in this study, your Study Doctor will decide how to continue to manage your breast cancer. If at that time you are receiving benefit from the study drug you may continue study drug treatment in a separate study.

### **What do I have to do?**

You must be willing to attend the scheduled visits. Participation will involve you making four additional visits to the hospital. The reason for these additional visits is that this is a new drug and we wish to carry out more regular assessment. For any additional visits to the hospital we will be happy to arrange either a taxi to pick you up and take you home or pay your travelling expenses. It is also important that you take the study medication as directed. Any left over study medication that you do not take, and the container even if it is empty, must be returned at each of your visits. It is also important that you

tell the medical staff about any other medication you are taking before and during the study.

If you are female, you must not be pregnant or breast-feeding and you must not become pregnant during the study. You should use acceptable methods of birth control (i.e., birth control pills, condoms, approved contraceptive implant, intrauterine device, or tubal ligation) throughout the study to prevent pregnancy. Your Study Doctor must be told immediately if pregnancy occurs. Males taking ZD1839 (GEFITINIB™) must also use birth control while taking the drug to avoid pregnancy of a partner. Acceptable methods include foam and condoms or vasectomy.

**What are the possible side effects, risks and discomforts of taking part?**

ZD1839 (GEFITINIB™) may cause some side effects. These are usually mild to moderate. Do not be alarmed by the list of side effects. You may experience none, some or all of those listed below.

Contact your Study Doctor promptly if any of the following happens to you, as you may need further examinations or treatment: diarrhoea; serious breathlessness, or sudden worsening breathlessness, possibly with a cough or fever; severe eye problems (some subjects have suffered from ulcer on the surface of the eye (cornea), sometimes with ingrowing eyelashes); extremely severe skin reactions with lesions, ulcers or blisters, skin sloughing or involvement of the lips and mucous membranes (toxic epidermal necrolysis, erythema multiforme). This type of skin reaction is rare, and is different to the more mild and more common skin reactions described below.

Other very common side effects (more than 10 of every 100 subjects are likely to have them): skin reactions such as acne-like rash, sometimes itchy with dry skin; mild to moderate diarrhoea; nausea (feeling sick).

Other common side effects (1 to 10 of every 100 subjects are likely to have them): vomiting; loss of appetite; red and sore mouth; nail problems; loss of hair; weakness; red and itchy eye; red and sore eyelid.

Take special care with ZD1839 (GEFITINIB™). If you get very breathless, or your breathlessness suddenly gets worse, possibly with a cough or fever, tell your doctor straight away. Some patients taking ZD1839 (GEFITINIB™) get an inflammation of the lungs called interstitial lung disease. This side-effect is uncommon (less than 1 in every 100 patients), and some of the patients have died from this. Your doctor may do some tests and may change your treatment.

Changes in the way your liver works may occur with ZD1839 (GEFITINIB™) and the function of your liver can be monitored by taking a blood test. If these blood tests become very high, your Study Doctor may need to stop the treatment.

There could be changes to the way your blood clots if you are taking warfarin (Coumadin) (medicine to prevent blood clotting). Blood tests will need to be done regularly to check the clotting time of your blood.

ZD1839 (GEFITINIB™) is not expected to impair your ability to drive or use machines. However, some subjects may occasionally feel weak. If this happens to you, do not drive or use machines.

There may be risks involved in taking this medication that have not yet been identified in the studies done so far. There is always a risk involved in taking a

new medication but every precaution will be taken and you are encouraged to report anything that is troubling you. The taking of a blood sample may cause some pain, bruising, light headedness, and on rare occasions, infection.

You understand you will have to notify you Study Doctor immediately of any unusual side effects.

**What are the possible benefits of taking part?**

It is hoped that the treatment will help you. However this cannot be guaranteed.

The information we get from this study may help us to treat future subjects with breast cancer better.

**What if new information becomes available?**

If any new information on the medications becomes available which may influence your decision to continue in the study you will be told.

**What happens if something goes wrong?**

We appreciate that patients may have complaints as to their treatment by members of staff (doctors, nurses etc) or they may have a complaint because something has happened during or following their participation in the trial – i.e. a reportable serious adverse event. Complaints from patients as to their treatment by members of staff should be addressed in the usual way to the Chief Executive of the hospital. If a patient suffers an adverse event due to the drug your doctor would wish to be informed of this as soon as possible.

If you are harmed due to someone's negligence then you may have grounds for a legal action but you may have to pay for it. Regardless of this if you wish to complain about any aspect of the way you have been approached or treated

during the course of this study the normal National Health Service complaints mechanisms are available to you.

Compensation for any injury caused by taking part in this study will be in accordance with the guidelines of the Association of the British Pharmaceuticals (ABPI). Broadly speaking the ABPI guidelines recommend that the sponsor, without legal commitment, should compensate you without having to prove that it is at fault. This applies in cases where it is likely that such injury results from giving any new drug or any other procedure carried out in accordance with the protocol for the study. The sponsor will not compensate you where such injury results from any procedure carried out which is not in accordance with the protocol of the study. Your right at law to claim compensation for injury where you can prove negligence is not affected. Copies of these guidelines are available on request.

**Will the information collected be confidential?**

If you consent to take part in the study, any of your medical records will be directly inspected by the company sponsoring the study, contractors working on behalf of the sponsoring company and may also be inspected by the Regulatory Authorities and/or the Independent Ethics Committee to check that the study is being carried out correctly. By signing the written informed consent form you are giving permission for this to be done.

The information collected during the study will be stored in a computer but your name will not be. The data and results from this study may also be presented at meetings or in publications, but in those presentations study participants will not be identified by name.

Data collected during the study, will be submitted to the company sponsoring the study and contractors working on behalf of the sponsor, and may be submitted to the Regulatory Authorities outside the European Economic Area for the purpose of safety and efficacy evaluation and approval to market the study medication.

**What will happen to the results of the research study?**

The results of the study will be initially presented at scientific meetings. Thereafter the results will be combined into a publication. It is possible that if ZD1839 (GEFITINIB™) is shown to be an effective new drug for breast cancer the results will be shown to the Regulatory Authorities.

**Who is organising and funding the research?**

The study has been proposed by Professor John Robertson. He has received an unrestricted investigational grant from the company who make ZD1839 (GEFITINIB™), AstraZeneca, to carry out this proposed study. The funding will be used to support the research nurse and one of the research doctors.

**Who should I contact if I need more information or help?**

In case of study-related injury or whenever you have questions about the study, or your study medication, please contact:

Professor J F Robertson (Phone Number: \*\*\* \*\*\*)

Mr K L Cheung (ext. \*\*\*\*\*), Mr R Cochrane (ext. \*\*\*\*\*)

Or any of the breast care research nurses in the department of surgery during the week or the Ward Sister or Staff Nurse on \*\*\*\*\* \* Ward (ext. \*\*\*\*\* or ext. \*\*\*\*\* at weekends).

## PATIENT INFORMED CONSENT

Study Number:        \_ \_ \_ \_ \_ / \_ \_ \_ \_ \_

Centre Number:        \_ \_ \_ \_ \_

Subject E-code:        E \_ \_ \_ \_ \_

Subject

Initials: \_ \_ \_

***Phase II trial to assess the efficacy of ZD1839 (GEFITINIB™) 500mg/day in patient with breast cancer who have either failed on tamoxifen or have an oestrogen receptor negative tumour and would be considered for systemic therapy.***

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**Please**

**initial box**

I,                    (Name                    of                    subject,                    in                    block letters)..... have read and understood all the information given to me about my participation in this study and I have been given the opportunity to discuss it and ask questions. All my questions have been answered to my satisfaction and I voluntarily agree to take part in this study. I understand that I will receive a copy of this Written Informed Consent form.

I authorize the release of my medical records to the sponsor (including its contractors), Regulatory Authorities and the Independent Ethics Committee.



I understand that the information I provide will be processed and analysed as is required by this clinical study and according to the Data Protection Act.

I have read and understand the subject information sheet which details how tissue sample from my tumour will be used and stored, and how the information from the tests will be used, and I understand that consent that I give now is for the lifetime of the sample and that refusal to consent to the research use of biological materials will in no way affect my clinical care.

The tissue samples from my tumour collected in this study, the results of any testing, and any patents, diagnostic tests, drugs, and biological products developed directly or indirectly as a result of this study, as well as any information derived directly or indirectly from those samples, is the sole property of the AstraZeneca (and its successors, licensees, and assigns). I have no right to this property or to any share any profits that may be earned directly or indirectly as a result of this study.

I agree to coded use of my biological materials for future non-genetic studies.

I consent to my GP being informed that I am in this study

I understand that I am free to withdraw from the study at any time,  
without having to give reason and without affecting my future  
medical care

I agree to take part in the above study.

\_\_\_\_\_  
\_\_\_\_\_

Name of Patient (block capitals)

Date

Signature

I have explained the nature and purpose of the study to the named patient  
above.

\_\_\_\_\_  
\_\_\_\_\_

Name of researcher taking consent (block capitals)

Date

Signature

(Principal Investigator or medically qualified delegate)

Copies: 3 (1 for patient; 1 for researcher; 1 to be kept with patient's notes)

### ***13.2 The Technique of Wide Needle Core Biopsy***

1. Introduce yourself to the patient. Explain the procedure and reassure them that once the local anaesthetic has been administered, the procedure will not be painful.
2. Ask the patient to remove the top half of their clothes and lie on a treatment couch with the back of the couch at 30 – 45 degrees to the horizontal.
3. Palpate the breast to locate the lump
4. Disinfect the skin with an appropriate antiseptic solution
5. Take 5 – 10 mls of 1% lignocaine with adrenaline and raise a bleb lateral to the lump. Infiltrate all around the lump.
6. Wait several minutes
7. Whilst waiting assemble the core biopsy gun. Advise the patient that the gun makes a sharp noise. Fire the gun several times to illustrate this.
8. Check that anaesthesia is adequate, using an 18 gauge green needle to touch the incision site
9. Using a small scalpel blade, make a small incision in the skin over the bleb, lateral to the lump.
10. Advance the core biopsy gun through the incision until it rests at the edge of the lump.
11. Stabilise the breast and lump with one hand, release the safety catch and fire the gun.
12. Withdraw the gun and slide the mechanism back to check that an adequate core of tissue has been obtained.

13. Place the core of tissue into either a cellsafe and formalin pot or a flask of liquid nitrogen if it is to be snap frozen.
14. Repeat the sampling until sufficient numbers of cores have been obtained (usually 4).
15. Between sampling and at the end of the procedure put pressure on the wound to reduce the chance of haematoma formation.
16. Apply a small dressing or sticking plaster
17. Advise the patient to continue to apply pressure for 5 – 10 minutes
18. Fill in the relevant histopathology request forms
19. Clear away all sharps
20. Give the patient an advice sheet about the procedure to take home

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